REMARKS

Claims 1-7, 10, 12-14, 19-20, 25, 27, 29-30, and 32-40 constitute the pending claims in the present application. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

With regard to item 1 of the Office Action, the Examiner states that the disclosure is objected to because of informalities. Applicants have corrected informalities throughout the disclosure; therefore Applicants respectfully request reconsideration and withdrawal of this objection.

Rejection based on 35 U.S.C. 112, second paragraph. Claims 1-18 and 31 are rejected under 35 U.S.C. 112, second paragraph, because the claims do not set forth any steps involved in the method/process. Applicants have cancelled claims 8, 9, 11, and 15-18 and amended claims 1-7, 10, and 12-14 such that they are now directed to a method for treating colorectal cancer. Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection based on 35 U.S.C. 112, second paragraph. Claims 1-31 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Applicants traverse this rejection to the extent it is maintained over the claims as amended.

The Examiner states that claims 1, 15, 16, 19-24, and 28-30 recite the limitation of "ester, amide or prodrug thereof" which includes the narrow limitations of "ester" and "amide" as well as the broad limitation of "prodrug". Applicants have cancelled claims 15, 16, 21-24, and 28 and amended claims 1, 19, 29, and 30 such that the term "prodrug" has been removed. Applicants respectfully point out to the Examiner that the term "prodrug" does not appear in claim 20. Applicants respectfully request reconsideration and withdrawal of this rejection.

The Examiner further states that the claims are indefinite for using the term "such as". Applicants have amended claim 2 such that the term "such as" has been removed. Claim 5 has been amended such that the terms "including" and "particularly" have been removed.

The Examiner states that claim 8 lacks antecedent basis because it recites "R^2 or R^3 comprises a chain of at least 3... heteroatoms..." which is not recited in any of the preceeding claims. Claim 8 has been cancelled, therefore this rejection is moot. Applicants respectfully request reconsideration and withdrawal of this rejection.

The Examiner further states that claim 10 is indefinite because "methylene" is a divalent group while R^{15} is a terminal group. Applicants removed the term "methylene" from claim 10. Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection based on 35 U.S.C. 101. Claims 1-18 and 31 are rejected under 35 U.S.C. 101 because the claimed recitation of a use, without setting forth any steps involved in the process, results in an improper definition of a process. As discussed above, Applicants have cancelled claims 8, 9, 11, 15-18, and 31and amended claims 1-7, 10, and 12-14 such that they are now directed to a method for treating colorectal cancer. Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection based on 35 U.S.C. 112 and 101. Claims 1-18, 28, and 31 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specifically asserted utility or a well established utility. Applicants traverse this rejection to the extent it is maintained over the claims as amended.

Applicants have amended claim 1 such that it is directed to a method for treating colorectal or breast cancer in a warm blooded animal in need of such treatment, which comprises administering to said animal an effective amount of a compound of formula (I). Applicants submit that the claims as amended are directed to a well-established utility. Applicants further submit that it was well known in the art at the time that aurora 2 (also known as BTAK and AIK1) was amplified and its kinase product overexpressed in breast and colorectal cancers, as described in Exhibits A-D (Exhibit A: Sen et al., Oncogene, 1997, 14(18) 2195-2200; Exhibit B: Bischoff et al., EMBO, 1998, 17(11) 3052-3065; Exhibit C: Zhou et al., Nat. Genet., 1998 20(2) 189-193; Exhibit D: Tanaka et al., Cancer Res., 1999, 59(9) 2041-2044). Applicants respectfully request reconsideration and withdrawal of this rejection.

Regarding item 8 of the Office Action, Applicants have amended the claims to correct the informalities cited by the Examiner throughout the claims. Applicants respectfully request reconsideration and withdrawal of this rejection.

Concerning item 9 of the Office Action, the claims have been amended to conform to proper dependent form. Specifically, claims 12 and 13 have been amended such that they are dependent only on claim 1. Claims 21, 23, and 24 are cancelled. Claims 27, 29, and 30 have been amended such that they depend only on claim 19. Applicants respectfully request reconsideration and withdrawal of this rejection.

<u>Rejection based on 35 U.S.C. 102(b).</u> Claims 23-25, 27, 29, and 30 are rejected under 35 U.S.C. 102(b) as being anticipated by Hudson (WO 96/09294). Applicants traverse this rejection to the extent it is maintained over the claims as amended.

Claims 23 and 24 have been deleted. Claim 25 is now dependent on novel claim 20, and claims 27, 29, are dependent on novel claim 19. Applicants therefore respectfully request reconsideration and withdrawal of this rejection.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945**, under Order No. ASZD-P01-599 from which the undersigned is authorized to draw.

Dated: September 14, 2004

Respectfully submitted,
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Exhibit A

Oncogene (1997) 14, 2195–2200

A putative serine/threonine kinase encoding gene *BTAK* on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines

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DNA amplification on chromosome 20q13 is commonly detected in breast cancer and correlates with poor prognosis. Definitive critical target genes on this amplicon have however, not yet been identified. We describe in this paper isolation of a novel gene named *BTAK*, encoding a putative member of protein serine/threonine kinase family localized on chromosome 20q13 that is amplified and overexpressed in breast tumor cell lines. *BTAK* maps close to the critical region of amplification defined earlier on this amplicon. Deduced amino acid sequence shows conservation of all the subdomains predicted in protein kinase super family. Translated *BTAK* peptide shows significant homology with previously cloned protein serine/threonine kinase encoding genes Ipl1 from *S cerevisae* and *aurora* from Drosophila, both shown to be functionally involved in normal chromosome segregation process. Our findings suggest that amplification and overexpression of *BTAK* may be playing a critical role in oncogenic transformation of breast tumor cells.

Keywords: breast cancer; gene amplification; protein kinase

Introduction

Gene amplification is one of the most common forms of genetic abnormality occurring in human breast cancer that is manifested cytogenetically in the form of abnormal chromosomal segments in tumor cells (Devilee *et al.*, 1994). Genes frequently reported to be amplified in breast cancer include c-*erb*B2, c-*myc*, cyclin D1 that are harbored on chromosomal segments 17q21, 8q24 and 11q13 respectively. Availability of the cloned probes for the amplified genes first helped detection of this genetic abnormality. Recent application of molecular cytogenetic techniques of chromosome microdissection and comparative genomic hybridization (CGH) have identified additional chromosomal sites of DNA amplification in human breast cancer cells (Guan *et al.*, 1994; Kallioniemi *et al.*, 1994).

Among the novel sites of amplification identified, chromosomal region 20q11-q13 was detected in about 12–18% of primary breast tumors and 40% of breast tumor cell lines. CGH analysis also revealed strong association between amplification of DNA around chromosome 20q13 region and poor prognosis in node negative breast cancer (Isola *et al.*, 1995). These data suggest that chromosome segment 20q11-q13 harbors one or more novel oncogene(s), whose overexpression due to amplification plays a critical role in progression of breast cancer. Attempts are therefore underway to map the common region of amplification spanning this chromosome segment in breast cancer cells and clone the amplified, overexpressed gene(s) harbored on this amplicon.

Interphase FISH analysis with anonymous cosmid probes and gene specific P1 clones earlier was reported to define approximately 1.5 Mb of minimum common region of amplification at chromosome 20q13.2 in breast tumor cells that excluded all candidate genes in the region from this amplicon (Tanner *et al.*, 1994). More recent FISH analysis with additional region specific cloned probes have however revealed that the amplification pattern of 20q chromosomal DNA in breast cancer cells is more complex and extends over a longer distance than previously reported (Tanner *et al.*, 1996; Sen *et al.*, unpublished results).

We have generated a YAC based physical map of the amplified region. These YACs are being used to identify expressed transcripts encoded on the amplified chromosome segment by the approach of direct cDNA selection. Direct cDNA selection has proved to be an effective method for isolating genes from several large genomic regions (Lovett *et al.*, 1991; Morgan, 1992; Osborne-Lawrence *et al.*, 1995). This methodology involves isolation of expressed sequences through the hybridization of a library or pool of cDNAs to a genomic target. In this paper we describe isolation, by direct selection, of a partial cDNA sequence for a putative serine/threnonine kinase encoding gene harbored on chromosome 20q13 that is amplified and overexpressed in human breast cancer cell lines. This novel gene being called *BTAK* (*B*reast *T*umor *A*mplified *K*inase) shares significant homology with the kinase domains of yeast and Drosophila kinase known to be involved in chromosome segregation (Francisco *et al.*, 1994; Glover *et al.*, 1995). *BTAK* gene maps close to the region on 20q amplicon that has been associated with aggressive breast cancer.

Results

To assess *BTAK* gene copy number in breast cancer cells, genomic DNAs from three tumor cell lines BT474, MCF7, SKBR3 and two control cell lines WI38,

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MCF10 were digested with restriction enzymes, electrophoresed through agarose gel and then Southern blot hybridized with *BTAK* cDNA probe. As shown in Figure 1a, all the three breast tumor cell lines revealed amplified signal intensities on genomic EcoRI fragments of about 8.0 kb and 5.5 kb sizes. Quantitation of amplification level was done by comparing the ratio of signal intensities between amplified *BTAK* hybridizing bands and a β actin hybridizing band among the tumor cell line and control cell line DNAs. This analysis revealed that *BTAK* gene is about $3\times$, $5\times$, and $8\times$ amplified in the breast tumor cells SKBR3, MCF7 and BT474 respectively.

The *BTAK* probe besides detecting amplified genomic fragments also consistently hybridized with an unamplified genomic fragment of about 6.1 kb size in all of the five cell line DNAs. This result indicated to us that *BTAK* probe cross hybridized to a member of a related sequence family that is not amplified in the tumor cells.

Steady state level of *BTAK* transcript was analysed in the same three breast tumor cell lines and the two control cell lines by Northern blot hybridization of their mRNA with *BTAK* cDNA probe. Distinct hybridization signal on mRNA of about 2.4 kb size was detected in all the cell types. There was no other heterogeneity in the transcript size revealed in these cells. Level of expression of the transcript was markedly higher in the breast tumor cell lines compared to the control cell lines. Among the cell lines, *BTAK* mRNA levels correlated with the extent of amplification of the gene detected in each case. Thus BT474 with highest gene copy number was found to express at the highest level while MCF7 and SKBR3 cells with progressively lower degree of amplification of the gene expressed the transcript at an intermediate and lowest level among the three cell lines (Figure 1b). Level of *BTAK* mRNA over expression was quantitated in the tumor cell lines by measuring the signal intensity of the *BTAK* mRNA band and normalizing the values for mRNA loading according to the β actin mRNA hybridization signal detected in each lane (Figure 1c). This analysis revealed that compared to the control cell lines, *BTAK* mRNA is expresssed at about threefold, sixfold and 10-fold excess in SKBR3, MCF7 and BT474 cells respectively.

BTAK gene was mapped to chromosome 20q by selective PCR amplification of a *BTAK* specific genomic fragment with DNA from human/rodent somatic cell hybrid cells containing either an intact or portion of human chromosome 20. Primers for PCR localization of *BTAK* were designed from a 260 bp repeat free region of the cloned *BTAK* specific 5.5 kb genomic fragment that showed sequence identity with the cDNA clone isolated.

As shown in Figure 2, of all the human/rodent hybrid cell DNAs, only the one containing chromosome 20 showed the expected 260 bp PCR product, thus demonstrating that this gene is localized on human chromosome 20. The lane with total human

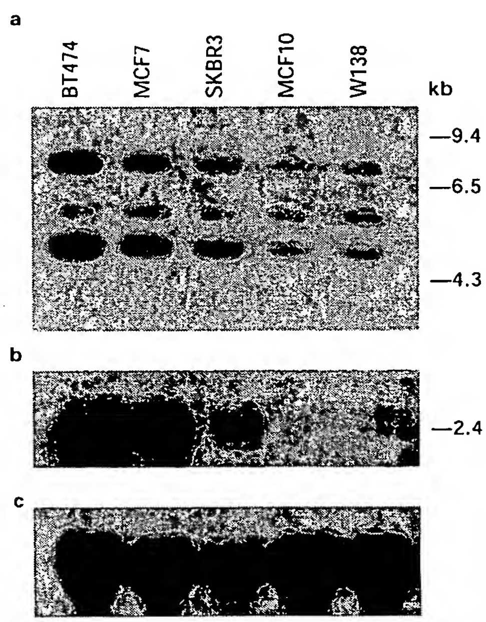

Figure 1 Southern (a) and Northern (b) blot analyses of breast tumor cell DNAs and RNAs with the pc*BTAK7* insert. Diploid human lung fibroblast cells WI38 and chemically transformed human mammary epithelial cells MCF10 were included as controls. Note progressively more amplified copy number of 8.0 kb and 5.5 kb EcoRI *BTAK* genomic fragments in SKBR3, MCF7 and BT474 breast tumor cells. *BTAK* probe cross hybridized with an unamplified 6.1 kb fragment also (a). *BTAK* mRNA overexpression in these cells is shown in (b). (c) shows comparable mRNA loading in each lane following hybridization with a β actin probe

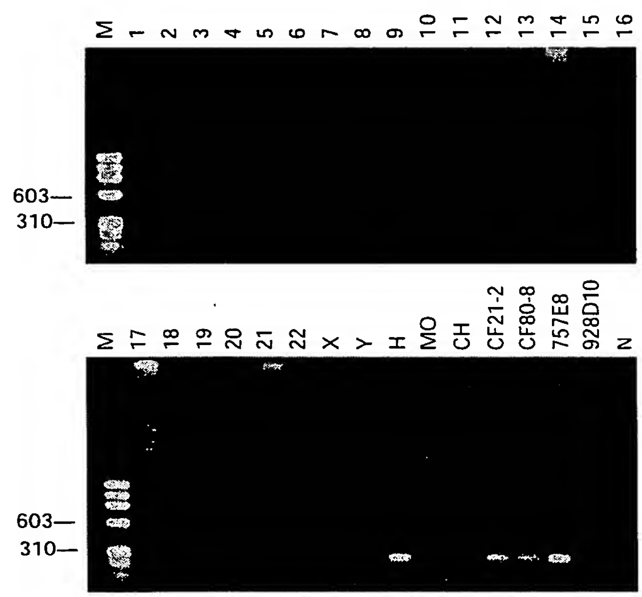

Figure 2 Chromosomal and YAC localization of the *BTAK* gene probe. Primers complementary to a 260 bp *BTAK* genomic sequence were utilized for PCR localization of the gene with DNA from a human/rodent somatic cell hybrid panel containing human chromosomes (1–22, X, Y). M represents the DNA molecular weight marker. Lanes with human chromosomes are accordingly marked. H, MO and CH represent lanes with parental human, mouse and Chinese hamster cell DNAs. CF21-2, CF80-8 are human/mouse hybrid cells containing human chromosome 20 cen-qter and 20q13.1-qter regions respectively. 757e8 and 928d10 are two YACs containing chromosome 20q DNA. N is the negative control set up for PCR without DNA. *BTAK* specific PCR product is visible with total human DNA, chromosome 20 containing hybrid DNAs and YAC 757e8 DNA

DNA also revealed the *BTAK* gene specific DNA fragment as expected. Amplification of this fragment in two additional hybrid cell DNAs containing fragments of chromosome 20 (CF21-2, CF80-8) helped to further narrow down the site of localization of *BTAK* gene to chromosome region 20q13. High resolution mapping of the gene was done by PCR screening of the YAC clones utilized for cDNA selection. Clone 757e8 was found positive for the 260 bp *BTAK* specific product. 757e8 is positive for the markers D20S100 and D20S102 which also flank the previously described critical region of amplification at 20q13.2 in breast cancer cells (Tanners *et al.*, 1994).

Sequencing of the two complementary strands of the insert in the cDNA clone pc*BTAK7* revealed that it contained 660 bp. Complete sequence of the cloned cDNA is shown in Figure 3. An open reading frame was identified which agreed with the highest scoring known coding sequences. Predicted amino acid sequence of the translated peptide corresponding to the open reading frame is shown with single letter amino acid symbols below the nucleotide sequence in the figure. The *BTAK* peptide sequence was analysed for similarity with known peptides available in the Gene Pept database. The four highest scoring sequences included two unpublished kinases p46x1Eg22 and p46Eg265 from *Xenopus laevis*, *aurora* protein kinase from Drosophila and *Ipl1* protein kinase from *S cerevisae* all belonging to serine/threonine family of kinases (Figure 4). *BTAK* revealed about 70% identity with the *X laevis* kinases and about 50% identity with the *aurora* and *Ipl1* kinases. One peptide from *X laevis* and the ones from Drosophila and *S cerevisae* were aligned with the deduced sequence from pc*BTAK7* using PILE UP and the ends terminated to match all the four sequences. Conserved amino acid residues for the kinase domain primary structure are also shown in the alignment as the consensus line in Figure 4. First 10 of the 12 subdomains recognized in the kinase domains could be assigned on the deduced amino acid sequence of the pc*BTAK7*. It is likely that the remaining subdomains will be identified when the full length sequence is isolated. Nine kinase domain residues recognized as invariant or nearly invariant throughout the kinase superfamily (Hanks and Hunter, 1995) were also found conserved in the deduced *BTAK* peptide.

```
  1  CAGTGGGCTTTGGAAGACTTTGAAATTGGTCGCCCTCTGGGTAAAGGAAAGTTTGGTAAT   60
     Q  W  A  L  E  D  F  E  I  G  R  P  L  G  K  G  K  F  G  N
 61  GTTTATTTGGCAAGAGAAAAGCAAAGCAAGTTTATTCTGGCTCTTAAAGTGTTATTTAAA  120
     V  Y  L  A  R  E  K  Q  S  K  F  I  L  A  L  K  V  L  F  K
121  GCTCAGCTGGAGAAAGCCGGAGTGGAGCATCAGCTCAGAAGAGAAGTAGAAATACAGTCC  180
     A  Q  L  E  K  A  G  V  E  H  Q  L  R  R  E  V  E  I  Q  S
181  CACCTTCGGCATCCTAATATTCTTAGACTGTATGGTTATTTCCATGATGCTACCAGAGTC  240
     H  L  R  H  P  N  I  L  R  L  Y  G  Y  F  H  D  A  T  R  V
241  TACCTAATTCTGGAATATGCACCACTTGGAACAGTTTATAGAGAACTTCAGAAACTTTCA  300
     Y  L  I  L  E  Y  A  P  L  G  T  V  Y  R  E  L  Q  K  L  S
301  AAGTTTGATGAGCAGAGAACTGCTACTTATATAACAGAATTGGCAAATGCCCTGTCTTAC  360
     K  F  D  E  Q  R  T  A  T  Y  I  T  E  L  A  N  A  L  S  Y
361  TGTCATTCGAAGAGAGTTATTCATAGAGACATTAAGCCAGAGAACTTACTTCTTGGATCA  420
     C  H  S  K  R  V  I  H  R  D  I  K  P  E  N  L  L  L  G  S
421  GCTGGAGAGCTTAAAATTGCAGATTTTGGGTGGTCAGTACATGCTCCATCTTCCAGGAGG  480
     A  G  E  L  K  I  A  D  F  G  W  S  V  H  A  P  S  S  R  R
481  ACCACTCTCTGTGGCACCCTGGACTACCTGCCCCCTGAAATGATTGAAGGTCGGATGCAT  540
     T  T  L  C  G  T  L  D  Y  L  P  P  E  M  I  E  G  R  M  H
541  GATGAGAAGGTGGATCTCTGGAGCCTTGGAGTTCTTTGCTATGAATTTTTAGTTGGGAAG  600
     D  E  K  V  D  L  W  S  L  G  V  L  C  Y  E  F  L  V  G  K
601  CCTCCTTTTGAGGCAAACACATACCAAGAGACCTACAAAAGAATATCACGGGTTGAATTC  660
     P  P  F  E  A  N  T  Y  Q  E  T  Y  K  R  I  S  R  V  E  F
```

Figure 3 Nucleotide sequence of the pcBTAK7 cDNA clone and the predicted amino acid sequence of the peptide corresponding to the open reading frame. Bar on nucleotides 476-652 show the sequence identified in the genomic clone isolated

```
                                           I                     II
              1            *********   **********   ******             50
btak          QWALEDFEIG   RPLGKGKFGN  VYLAREKQSK   FILALKVLFK  AQLEKAGVEH
p46xleg22     QWCLEDFEIG   RPLGKGKFGN  VYLARERESK   FILALKVLFK  SQLEKAGVEH
aurora        TWELNNFDIG   RLLGRGKFGN  VYLAREKESQ   FVVALKVLFK  RQIGESNVEH
ipl1          SLSLDDFELG   KKLGKGKFGK  VYCVRHRSTG   YICALKVMEK  EEIIKYNLQK
Consensus     QW-LEDFEIG   RPLGKGKFGN  VYLARE-ESK   FILALKVLFK  -Q-EKA-VEH
            III                      IV            V
              51                                      .               100
btak          QLRREVEIQS   HLRHPNILRL  YGYFHDATRV   YLILEYAPLG  TVYREL..QK
p46xleg22     QLRREVEIQS   HLRHPNILRL  YGYFHDASRV   YLILDYAPGG  ELFREL..QK
aurora        QVRREIEIQS   HLRHPHILRL  YAYFHDDVRI   YLILEYAPQG  TLFNALQAQP
ipl1          QFRREVEIQT   SLNHPNLTKS  YGYFHDEKRV   YLLMEYLVNG  EMYKLLRLH.
Consensus     QLRREVEIQS   HLRHPNILRL  YGYFHD--RV   YLILEYAP-G  -L-REL--QK
                                   VIA                    VIB         VII
              101                           ****   **********         150
btak          LSKFDEQRTA   TYITELANAL  SYCHSKRVIH   RDIKPENLLL  GSAGELKIAD
p46xleg22     CTRFDDQRSA   LYIKQLAEAL  LYCHSKKVIH   RDIKPENLLL  GSNGELKIAD
aurora        MKRFDERQSA   TYIQALCSAL  LYLHERDIIH   RDIKPENLLL  GHKGVLKIAD
ipl1          .GPFNDILAS   DYIYQIANAL  DYMHKKNIIH   RDIKPENILI  GFNNVIKLTD
Consensus     --RFD-QRSA   TYI-QLANAL  LY-HSK--IH   RDIKPENLLL  GSNG-LKIAD
                                            VIII         IX
              151                                                     200
btak          FGWS.VHAPS   SRRTTLCGTL  DYLPPEMIEG   RMHDEKVDLW  SLGVLCYEFL
p46xleg22     FGWS.VHAPS   SRRTTLCGTL  DYLPPEMIEG   RMHDEKVDLW  SLGVLCYEFL
aurora        FGWS.VHEPN   SMRMTLCGTV  DYLPPEMVQG   KPHTKNVDLW  SLGVLCFELL
ipl1          FGWSIINPPE   NRRKTVCGTI  DYLSPEMVES   REYDHTIDAW  ALGVLAFELL
Consensus     FGWS-VHAPS   SRRTTLCGT-  DYLPPEM-EG   RMHDEKVDLW  SLGVLC-E-L
              201                      222
btak          VGKPPFEANT   YQETYKRISR  VEF
p46xleg22     VGKPPFETDT   HQETYRRISK  VEF
aurora        VGHAPFYSKN   YDETYKKILK  VDY
ipl1          TGAPPFEEEM   KDTTYKRIAA  LDI
Consensus     VGKPPFE--T   Y-ETYKRISK  V-P
```

Figure 4 Alignment of deduced *BTAK7* peptide with homologous 46 × leg22, *aurora* and *Ipl1* peptides available in Gen Pept data base using PILEUP. Conserved protein kinase specific subdomains are indicated by Roman numerals. Kinase domain specific invariant or nearly invariant residues conserved throughout the superfamily are shown in bold. The asterisks denote the protein serine/threonine motifs from the ProSite Dictionary of Bairoch

Discussion

We have isolated a novel human gene named *BTAK* localized on chromosome 20q13 that is amplified and overexpressed in breast cancer cell lines.

Recent FISH studies of breast tumor cells with P1 and cosmid probes defining genes and loci along 20q have demonstrated that in addition to the previously described critical region of amplification on 20q13 (Tanner *et al.*, 1994), two other non syntenic regions, 3 Mb and 20 Mb proximal spanning the segments 20q11 and 20q12 are also amplified in breast tumor cells (Tanner *et al.*, 1996). These results are in agreement with those obtained by our group also. Among the expressed sequences localized on these regions, vitamins D hydroxylase (CYP24) gene and a cellular apoptosis susceptibility (CAS) gene (Brinkman *et al.*, 1996) on 20q13, as well as the PTP1B/PTPN1 gene encoding a nonreceptor tyrosine phosphatase on 20q12 overexpressed in breast carcinomas (Wiener *et al.*, 1994) have been implicated as critical candidate genes on these amplicons. Recently, however, low level amplification of CAS gene in only selected cell lines and primary tumors has been cited as an evidence against this gene being the target for 20q amplification. Similarly, due to lack of correlation between amplification, and overexpression for the remaining genes on 20q amplicon, these have also been excluded as amplified targets in breast tumor cells (Tanners *et al.*, 1996). Two recently isolated expressed sequences A1B3

and A1B4 on chromosome 20q are yet to be proven definitive targets on the respective amplicon. (Guan *et al.*, 1996). *BTAK* gene, being described in this paper maps close to the critical region of amplification defined on 20q13 and shows good correlation between amplification and overexpression in all the three breast tumor cell lines analysed. Further, level of amplification detected on Southern blots in the three cell lines corraborated earlier findings reported in the same cells for genomic probes spanning the critical region based on quantitation of fluorescence *in situ* hybridization (FISH) signals or interphase nuclei (Tanner *et al.*, 1994). The *BTAK* gene therefore appears to be a strong candidate for being the target on the 20q13 amplicon. FISH studies done in our laboratory with appropriate genomic probes on primary tumor cells support this possibility. Amplification of chromosome 20q DNA has been detected in colon and bladder cancer cells by CGH technique (Kallioniemi *et al.*, 1994). 20q amplification has also been reported to accompany human papilloma virus 16/E7 mediated transformation of human uroepithelial cells which undergo immortalization and polyploidization at early passages (Reznikoff *et al.*, 1994). In addition to these molecular cytogenetic data, elevated copy number of chromosome 20 has been implicated in several cancer cell types based on their karyotypic analyses. These include lung cancer, gliomas, melanoma, ovarian cancer and neuroblastoma (Mitelman, 1991; Hay *et al.*, 1992). It would be of interest to find out how many of these malignant cell types contain the amplified and overexpressed *BTAK* gene.

Sequence analyses of the partial cDNA clone isolated revealed that the *BTAK* gene encodes a putative member of protein serine/threonine kinase family. Predicted peptide showed conservation of the consensus serine/threonine kinase signature sequences and all the expected subdomain specific residues that are implicated as playing essential roles in enzyme function of this family of proteins. This partial *BTAK* peptide sequence also revealed high degree of homology to previously cloned members of the serine/threonine kinase family. Two members of this protein family called *Ipl1* from *S cerevisae* and *aurora* from Drosophila have been shown to be involved in the regulation of high fidelity chromosome segregation process. Temperature sensitive conditional *S cerevisae IplI* mutants missegregate chromosomes at restrictive temperature (Chan and Botstein, 1993). Mutations in the gene *aurora* in Drosophila have been reported to give rise to chromosome segregation abnormalities ranging from generation of polyploid nuclei to mitotic arrest depending on the allelic variants of the locus involved (Glover *et al.*, 1995). Significant sequence homology shared by *Ipl1*, *aurora* and *BTAK* suggests that these proteins belong to a family of conserved protein serine/threonine kinases which are involved in regulation of chromosome segregation process. Phosphorylation and dephosphorylation of serine and threonine residues of proteins are known to be involved in the control of diverse cellular processes including chromosome segregation (Edelman *et al.*, 1987; Cohen, 1989). The phosphorylation state of a given protein depends on the relative activities of the protein kinase(s) and phosphatase(s) that recognize it as a substrate. It would therefore be important to identify the physiological substrate for the overexpressed *BTAK* kinase in breast cancer cells to determine how that is involved in the oncogenic transformation process.

Materials and methods

Cell lines, DNAs

Human breast cancer cell lines BT474, MCF7, SKBR3, human breast epithelial cell line MCF10 and diploid human lung fibroblast cell line WI 38 were all obtained from the American Type Culture Collection. Rodent/Human somatic cell hybrid panel containing specific human chromosomes were purchased from Corriell Mutant Cell Repository (Camden, NJ). DNA from two additional hybrids containing parts of human chromosome 20 (CF 21-2 with 20 cen-qter and CF 80-8 with 20q13.1-qter) were kindly provided by Dr DW Bowden of the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, North Carolina.

YAC clones spanning the physical map of the 20q amplicon in breast cancer cells were obtained from the Human Genome Center at Baylor College of Medicine, Houston and from Centre d'Etude du Polymorphism (CEPH), Paris. Recombinant plasmids containing 28S and 18S RNA genes were kind gifts from Dr Lisa Shaffer of the Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, Texas.

cDNA direct selection

YAC DNAs utilized for direct selection were 761c3, 757e8, 886f11, 845f3 and 847g7 spanning the 20qdis. amplicon mapped in BT474 cells (Sen *et al.*, manuscript in preparation). cDNA selection was performed according to the published method (Lovett, 1994) with some modifications.

YAC DNA preparation

To isolate YAC insert DNAs, agarose embedded yeast plugs were subject to pulsed field gel electrophoresis in a TAFE gel apparatus (Beckman Instruments). Recombinant YAC with human DNA insert was identified by Southern blot hybridization of the YAC DNAs with ^{32}P-labeled total human DNA. Insert containing YAC DNA was purified from another gel run under identical conditions. Gel purified YAC DNA was digested with Sau3A I restriction enzyme and ligated to a linker adapter sequence. The linker adapter sequence consisted of one 22 mer 'L' sequence 5'-GATCCCATGGTCGACTCGAGTC-3' and a complementary 18 mer 'S' sequence in opposite orientation, 5'-GACTCGAGTCGACCATGG-3' with Sau3A I compatible 5' cohesive termini at the end. Linker adapter ligated YAC DNAs were amplified by PCR in presence of the 'S' primer for 30 cycles with each cycle consisting of 94°C denaturation for 1 min, 50°C annealing for 1 min and 72°C extension for 2 min.

cDNA synthesis

A cDNA library with mRNA from BT474 cells was made in λgt11 vector utilizing the Superscript Choice System for cDNA synthesis (Gibco BRL) according to the manufacturer's manual. First strand cDNA synthesis was primed using a combination of oligo(dT) and random hexamer sequence in the presence of modified Moloney Murine Leukemia virus reverse transcriptase provided in the kit. cDNA inserts from the library were amplified by PCR in presence of the λgt11 cDNA insert screening amplimers (Clonetech). PCR was carried out for 30 cycles with each

cycle consisting of a 94°C denaturation step for 45 s, 60°C annealing step for 45 s, and a 72°C extension step for 2 min.

For selection of cDNAs, 2 μg of denatured YAC DNAs were applied onto nylon membranes using a slot blot apparatus (S&S Minifold II). Membrane was prehybridized with 50 μg of Cot 1 DNA in 1 ml hybridization buffer ($5 \times$ SSC, 0.05 M sodium phosphate, $5 \times$ Denhardt's, 1% SDS, 100 μg denatured sheared salmon sperm DNA) at 65°C for 4 h. At the same time to preblock repeat and nonspecific hybridization of cDNA sequences, 2 μg of PCR amplified BT474 cDNA was prehybridized with 2 μg of Cot 1 DNA, 100 ng of ribosomal DNA, 10 ng of ρYAC 4 vector DNA, 100 ng of yeast DNA in 200 μl of hybridization buffer at 65°C for 4 h. The DNA mix was then added to the membranes with YAC DNA and hybridization allowed to go for overnight. Membranes were then washed with $2 \times$ SSC, 0.1% SDS at room temperature followed by $0.1 \times$ SSS, 0.1% SDS at 65°C. cDNA hybridizing with the target YAC insert DNA was then eluted by incubating the membranes in 100 μl of boiling water for 10 min. Primary selected cDNA inserts were amplified with PCR and the selection procedure was repeated one more time. Secondary, selected cDNA amplified by PCR was cleaved with EcoRI and ligated to EcoRI digested λgt11 vector arms, packaged *in vitro* and plated.

Recombinant plaques were plated at a density of about 200 per 150 mm plate and transferred to Hybond N transfer membranes (Amersham). Filters were hybridized with radiolabeled total human DNA to eliminate the transcripts from intermediate repeats and from other highly expressed sequences such as ribosomal genes. Phages that failed to hybridize were isolated and analysed as described below.

Inserts from individual clones were obtained by PCR amplification. Inserts were pooled into groups of four and each pool was used as one probe on Northern blots containing RNA from control breast epithelial cell line MCF10 and breast tumor cell line BT474. It was rationalized that if one amplified gene encoded transcript in the pool expressed at a level four times or higher in BT474 cells than its single copy homologue in MCF10 cells, signal intensity for the amplified gene encoded transcript in BT474 RNA lane would appear equal or stronger than that detected in MCF10 RNA lanes. Among the first nine pools (36 independent clones) isolated and screened in this manner, one pool led to isolation of the cDNA clone being described in this paper. Hybridization analyses of the four probes from this pool, separately on four different Northern blots, identified one clone with an insert size of about 0.7 kb that detected a distinctly overexpressed transcript of about 2.4 kb in the BT474 cells.

Southern and Northern blot analysis

Southern and Northern blot hybridization analyses of DNA and RNA were performed according to standard protocols (Sambrook *et al.*, 1989) as described by us earlier (Sen *et al.*, 1994). Probes were labeled with [α-^{32}P]dCTP by random priming utilizing the random primed DNA labeling kit (Boehringer Mannheim).

Isolation of 5.5 kb BTAK *genomic clone*

Since Southern blot hybridization of EcoRI digested breast tumor cell DNAs with *BTAK* cDNA probe revealed an unamplified fragment in addition to the amplified ones, we decided to isolate the amplified *BTAK* specific genomic sequences from a size fractionated BT474 genomic DNA sublibrary. In order to isolate the *BTAK* specific amplified 5.5 kb EcoRI fragment, a genomic sublibrary of EcoRI digested 5–6 kb DNA was made in λgt11 vector following purification of the gel fractionated DNA with Gene CAPSULE (Midwest Scientific). 1×10^5 p.f.u. were screened with radiolabelled *BTAK* cDNA probe. Clone containing 5.5 kb *BTAK* genomic sequence was isolated. Restriction enzyme digestion and Southern blot hybridization of the genomic clone with the cDNA probe identified a 1.0 kb EcoRI–SmaI fragment sharing sequence identity between the two. This 1.0 kb EcoRI–SmaI fragment was subcloned in pBlue Script (pBS) and sequenced.

DNA sequencing and computational analyses

DNA sequencing was done by the Dideoxy chain termination method using the Seqnanase Version 2.0 DNA sequencing kit (United Stated Biochemical) manually in the laboratory and also on an Applied Biosystems Model 373A Automated DNA sequencer available as a core sequencing facility in our institution.

Computer analyses of the sequence was done using the Genetics Computer Group Package (Madison, WI). The GenBank database (Release 96.0) was searched using the BLAST software package (Altschull *et al.*, 1990). A second search was made over the GenPept database to identify known peptide sequences. As an additional approach *BTAK* peptide was analysed with the MOTIFS program to identify defined motifs.

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Exhibit B

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A homologue of *Drosophila aurora* kinase is oncogenic and amplified in human colorectal cancers

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Genetic and biochemical studies in lower eukaryotes have identified several proteins that ensure accurate segregation of chromosomes. These include the *Drosophila* aurora and yeast Ipl1 kinases that are required for centrosome maturation and chromosome segregation. We have identified two human homologues of these genes, termed *aurora1* and *aurora2*, that encode cell-cycle-regulated serine/threonine kinases. Here we demonstrate that the *aurora2* gene maps to chromosome 20q13, a region amplified in a variety of human cancers, including a significant number of colorectal malignancies. We propose that *aurora2* may be a target of this amplicon since its DNA is amplified and its RNA overexpressed, in more than 50% of primary colorectal cancers. Furthermore, overexpression of *aurora2* transforms rodent fibroblasts. These observations implicate *aurora2* as a potential oncogene in many colon, breast and other solid tumors, and identify centrosome-associated proteins as novel targets for cancer therapy.

Keywords: 20q13 amplicon/centrosome/colon cancer/oncogene/serine-threonine kinase

Introduction

Chromosomal abnormalities are a hallmark of human cancer, reflecting the deleterious consequences of the gain or loss of genetic information (Boveri, 1929; Hartwell and Kastan, 1994; Mitelman *et al.*, 1997). Some of these defects may have a causal role in cellular transformation due to loss of a negative growth regulator or a gene responsible for maintenance of genome integrity, or through the amplification, overexpression or mutational activation of an oncogene (Hunter, 1997; Kinzler and Vogelstein, 1997). Alternatively, these abnormalities may be a consequence of tumor progression, where disruption of mitotic checkpoints can result in abnormal nuclei, missegregated chromosomes and aneuploidy (Elledge, 1996; Sherr, 1996).

A direct connection between the cell cycle and cancer was first established with the observation that the cyclin D1 gene was amplified in a subset of human cancers (Motokura *et al.*, 1991; Hunter and Pines, 1994). The subsequent discovery that the tumor suppressor p53 regulates p21, an inhibitor of cyclin-dependent kinases (el-Deiry *et al.*, 1993; Xiong *et al.*, 1993), as well as the identification of p16—another cyclin-dependent kinase inhibitor—as a major tumor suppressor gene (Elledge, 1996; Sherr, 1996), has firmly entrenched the view that misregulation of the cell cycle machinery can have enormous impact on cellular proliferation. Based on the prevalence of genetic abnormalities in human cancer, it is plausible that proteins involved in maintaining the integrity of chromosome segregation may also play a role, directly or indirectly, in cellular transformation. The fidelity of chromosome segregation is monitored by mitotic checkpoints that delay entry into mitosis until a functional centrosome is present, or delay progression beyond anaphase until the chromosomes are aligned on the metaphase plate by the mitotic spindle. In normal cells, centrosomes play an important role in coordinating the changes required for the onset of mitosis, serving as an anchor for reorganization of the cytoplasmic microtubules into a mitotic spindle apparatus and for recruitment of numerous structural, motor and catalytic proteins to the centrosome complex. Proper execution of this process ensures that each daughter cell receives the correct number of chromosomes. Recent studies suggest that a G_2/M checkpoint may exist to ensure the integrity of this process of centrosome maturation (Nigg *et al.*, 1996).

Genetic and biochemical studies in yeast and *Drosophila* have identified several proteins involved in chromosome segregation and spindle assembly. Disruption of these proteins results in chromosome missegregation, monopolar or disrupted spindles and/or abnormal nuclei. Several of these proteins represent distinct families of protein serine/threonine kinases, including: Cdc2, a cyclin-dependent kinase conserved from yeast to mammals that is required for centrosome separation and formation of a bipolar spindle (Sherr, 1994, 1996; Morgan, 1995; Elledge, 1996); Mps1, a *Saccharomyces cerevisiae* dual specificity kinase required for spindle pole body duplication (Weiss and Winey, 1996); Bub1, a *S.cerevisiae* and mammalian mitotic checkpoint kinase (Hoyt *et al.*, 1991; Taylor and McKeon, 1997); PLK1, a mammalian homologue of polo, Cdc5p and plo1 kinases from *Drosophila*, budding and fission yeast, respectively, that communicates the presence of a functional centrosome to the Cdk/cyclin complex prior to entry into mitosis (Lane and Nigg, 1996, 1997); and the Ipl1 and *aurora* kinases from *S.cerevisiae* and *Drosophila*, respectively, that are required for centrosome

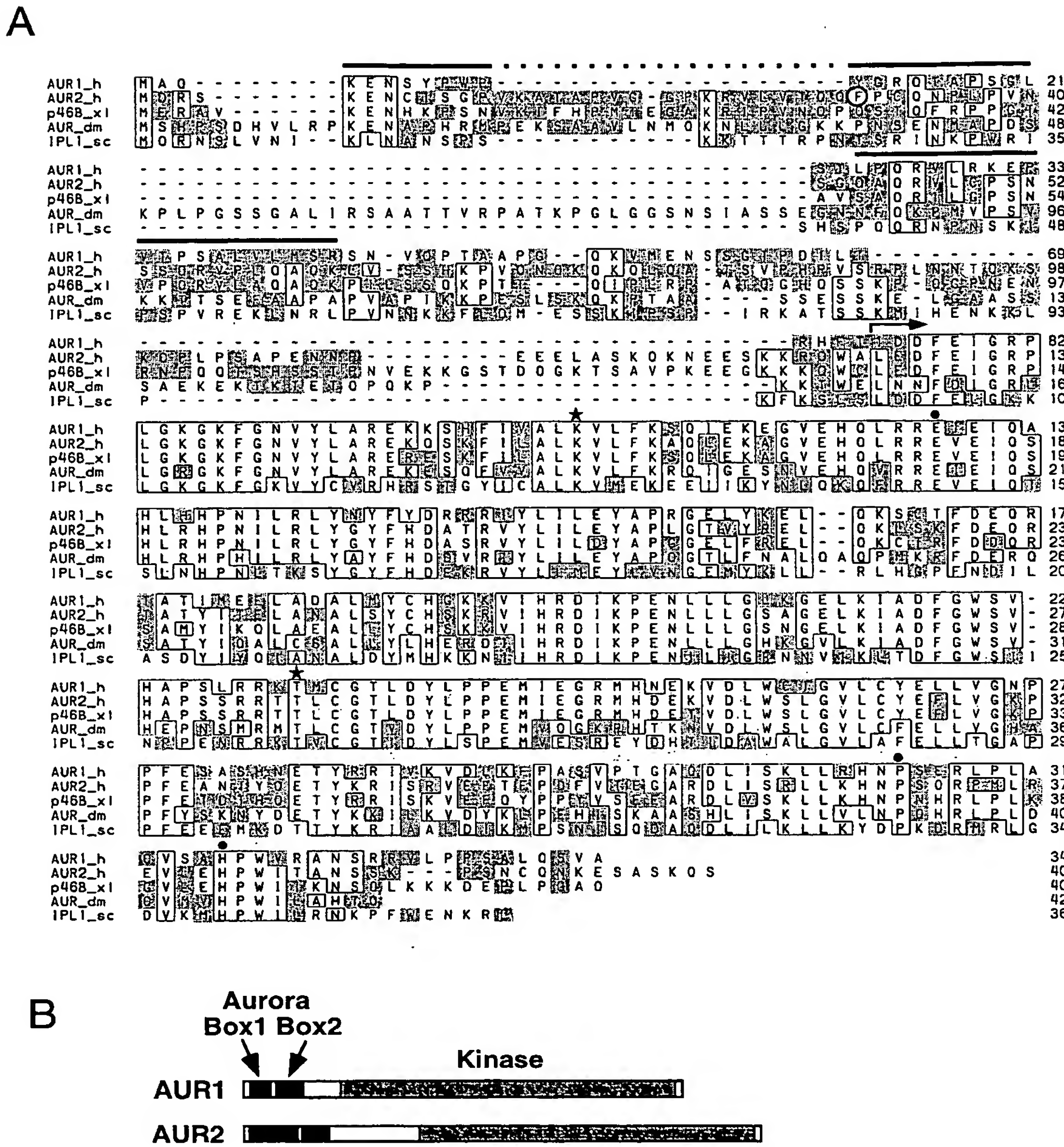


Fig. 1. Homology between human, *Xenopus*, *Drosophila* and yeast auroras. **(A)** The sequences for human aurora1 and aurora2 were deduced from full-length cDNA clones isolated from normal duodenum, pancreatic carcinoma and primary colorectal carcinoma libraries. *Xenopus* p46B (PIR:S53343), *Drosophila* aurora (PIR:A56220) and *S.cerevisiae* Ipl1 (SWISS-PROT:P38991) are included in the alignment. The alignment was generated by also including the two murine (DDBJ/EMBL/GenBank accession Nos D21099 and GB:U80932), an additional *Xenopus* (PIR:S53342) and two *C.elegans* (DDBJ/EMBL/GenBank accession Nos U53336 and U97196) sequences as input into msa, a parallel-coded multiple sequence alignment program that was run on MasPar MP2216 supercomputer. Boxed residues are common to three or more of the sequences; shaded residues represent regions of amino acid similarity between two or more sequences; overlines correspond to the conserved Aurora Box1 and Aurora Box2 sequences; the arrow denotes the start of the C-terminal serine/threonine kinase domain; the circled residue indicates the location of a single nucleotide polymorphism described in the text; solid circles correspond to the location of various yeast and *Drosophila* mutants (Franscisco *et al.*, 1994; Glover *et al.*, 1995); and stars denote the site of the kinase-inactivating and -activating point mutants described in the text. **(B)** Schematic domain structure of human aurora1 and aurora2.

separation and chromosome segregation (Francisco *et al.*, 1994; Glover *et al.*, 1995). Among these kinases, only PLK1 has been shown to be transforming (Smith *et al.*, 1997), although many are implicated to play a role in the genotypic changes associated with immortalized cells, possibly due to the presence of a compromised checkpoint (Hoyt *et al.*, 1991; Lane and Nigg, 1996, 1997; Taylor and McKeon, 1997).

Here, we describe the identification and characterization of two human homologues of *Drosophila* aurora and yeast Ipl1, that we have named aurora1 and aurora2.

Results

Structural comparison of aurora homologues

We initiated a PCR-based screen in order to identify novel colon cancer-associated kinases. One of these clones encoded a protein with homology to the aurora protein kinase from *Drosophila melanogaster* and the Ipl1 kinase from *S.cerevisiae* (Francisco *et al.*, 1994; Glover *et al.*, 1995). While using this fragment to screen for a full-length cDNA clone, we also identified a weakly hybridizing clone that was found to encode a related kinase. We refer to these genes as *aurora1* and *aurora2*, to reflect their homology to each other and to the *Drosophila aurora* kinase. The *aurora1* cDNA contained a 1032 bp open reading frame that encodes a 344 amino acid polypeptide with a predicted molecular mass of 39.3 kDa. The *aurora2* cDNA contained a 1209 bp open reading frame that encodes a 403 amino acid polypeptide with a predicted molecular mass of 45.8 kDa. Two additional human *aurora* pseudogenes were also identified as expressed transcripts that are each contained on single exons and maintain striking DNA homology to either *aurora1* or *2*, yet exhibit multiple frame shifts (G.D.Plowman, unpublished). During preparation of this manuscript, a partial sequence of *BTAK* (Sen *et al.*, 1997), a breast tumor-associated kinase, was reported that appears to be a fragment of human *aurora2*. A second paper reported the sequence of human *aik* (Kimura *et al.*, 1997), a cell cycle-regulated protein localized to spindle pole bodies. The published sequence shares 92% amino acid identity with our sequence of

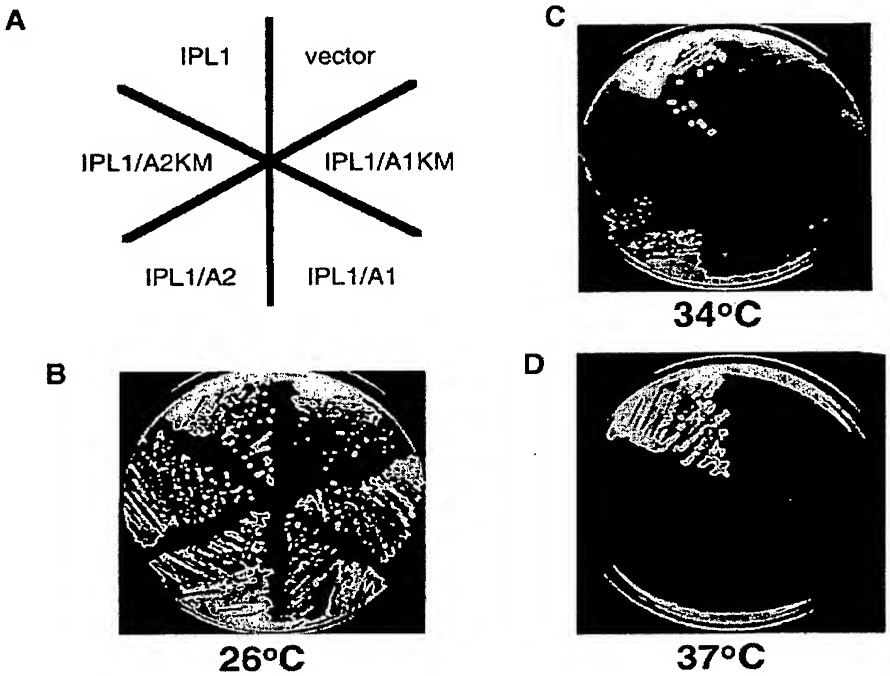
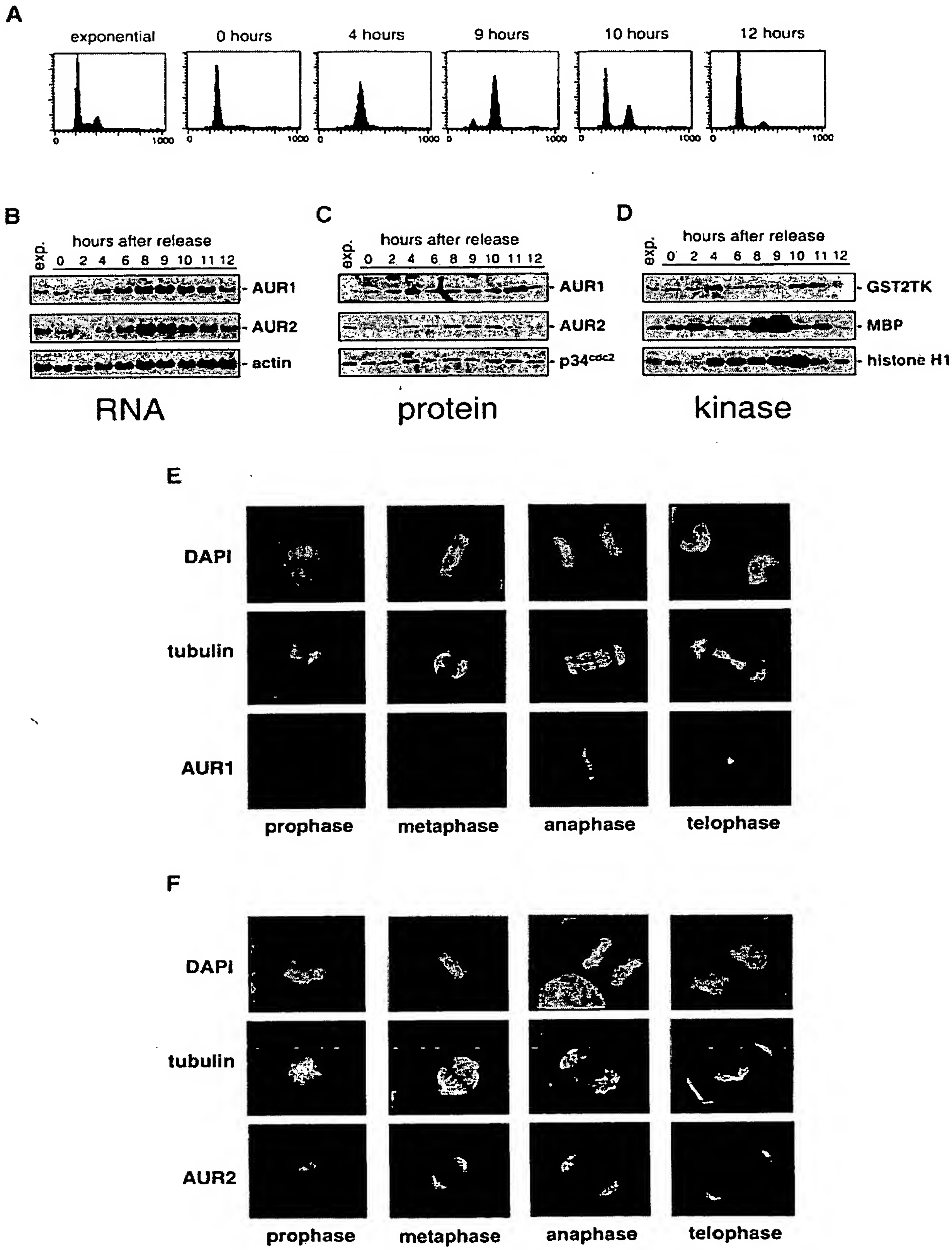


Fig. 2. The catalytic domain of aurora2, but not aurora1, partially complements the yeast *ipl1-1* mutation. (**A**) Map of various yeast transformants of strain CCY464-1D streaked onto SC-URA plates. Clockwise from top: vector, empty expression vector; Ipl1/A1KM, N-terminal domain of Ipl1 fused with the C-terminal portion of a kinase-dead *aurora1* construct; Ipl1/A1, N-terminal domain of Ipl1 fused with the C-terminal portion of wild-type *aurora1*; Ipl1/A2, N-terminal domain of Ipl1 fused with the C-terminal portion of wild-type *aurora2*; Ipl1/A2KM, N-terminal domain of Ipl1 fused with the C-terminal portion of a kinase-dead *aurora2* construct; and Ipl1, wild-type Ipl1. (**B**) Plate grown at the permissive temperature of 26°C. (**C**) Plate grown at the restrictive temperature of 34°C. (**D**) Plate grown at the restrictive temperature of 37°C.

Fig. 3. Aurora1 and aurora2 proteins are cell cycle-regulated and localized to mitotic structures. Exponentially growing HeLa cells were synchronized at the G_1/S transition by a double thymidine/aphidicolin block. Separate plates (10 cm) were harvested for FACS analysis, RNA isolation, protein quantitation and kinase assays at the indicated times. (**A**) FACS analysis was performed on exponentially growing HeLa cells, as well as cells harvested at 0, 4, 9, 10 and 12 h after release. (**B**) Northern blots of synchronized HeLa cells probed with a ^{32}P-labeled *aurora1* cDNA (top panel), a ^{32}P-labeled *aurora2* cDNA (middle panel), and a ^{32}P-labeled actin cDNA (bottom panel). Equal amounts of total RNA (10 μg) were loaded in each lane. (**C**) Immunoblots probed with protein A-purified anti-aurora1 antibodies (top panel), anti-aurora2 antibodies (middle panel) or anti-p34^{cdc2} antibodies (bottom panel). Equal amounts of total cellular protein (50 μg) were loaded in each lane. (**D**) *In vitro* kinase assays with anti-aurora1 immune complexes (top panel) using GST2TK (PKA phosphorylation site) as a substrate, with anti-aurora2 immune complexes (middle panel) using myelin basic protein (MBP) as an artificial substrate, or anti-p34^{cdc2} immune complexes (bottom panel) using histone H1 as a substrate. Equal amounts of total HeLa cell protein (500 μg) were used for each immunoprecipitation. (**E**) Aurora1 is localized to the midzone and post-mitotic bridge. HeLa cells at various stages of mitosis were stained for DNA, α-tubulin and aurora1. Top panels, DAPI staining of DNA; middle panels, α-tubulin immunostaining; bottom panel, aurora1 immunostaining. (**F**) Aurora2 is localized to the mitotic spindle of metaphase and anaphase cells. HeLa cells at various stages of mitosis were stained for DNA, α-tubulin and aurora2. Top panels, DAPI staining of DNA; middle panels, α-tubulin immunostaining; bottom panel, aurora2 immunostaining.


A
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RNA
C
hours after release
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hours after release
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0 2 4 6 8 9 10 11 12
GST2TK
MBP
histone H1
kinase
E
DAPI
tubulin
AUR1
prophase
metaphase
anaphase
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F
DAPI
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telophase

human aurora2. We believe that aurora2 and aik are identical and six frameshifts resulting from sequencing errors explain the small differences in the published sequence. Three additional papers provide the sequence of *AYK1* (Yanai *et al.*, 1997), a meiotic-regulated gene and *IAK1* (Gopalan *et al.*, 1997), both of which appear to be the murine orthologues of *aurora2*, and *AIM-1* (Terada *et al.*, 1998) which is a rat orthologue of *aurora1*. The current report describes the first complete sequence for both human *aurora1* and *aurora2*.

The deduced amino acid sequences of human aurora1 and aurora2 are presented in Figure 1A, aligned with the yeast and *Drosophila* homologues Ipl1 and aurora and an additional homologue p46B from *Xenopus laevis*. Human aurora2 protein shares 57%, 63%, 43% and 41% identity over its entire length with human aurora1, *Xenopus* p46B, *Drosophila* aurora and Ipl1, respectively. The four sequences contain a C-terminal domain with all the subdomains characteristic of a serine/threonine kinase. The kinase domain of human aurora2 shares 74%, 62% and 49% amino acid identity with human aurora1, *Drosophila* aurora and Ipl1, respectively, and 83.5% identity with two amphibian homologues present in *Xenopus* [p46A (p46Eg22, PIR:S53342) and p46B (p46Eg265, PIR: S53343)]. *Drosophila* aurora is most related to human aurora1 whereas yeast Ipl1 is most related to aurora2. Whereas a single aurora-like kinase is present in yeast, at least two members are present in *Caenorhabditis elegans* (DDBJ/EMBL/GenBank accession No. U53336, gene K07C11.2 and U97196, gene B0207.4). The deduced catalytic domains of these *C.elegans* proteins share 55% and 64% amino acid sequence identity to the human aurora2 kinase domain. We predict that an additional *aurora* homologue will ultimately be identified in *Drosophila* as characterization of its genome nears completion.

The 129 and 73 amino acid N-terminal domains of human aurora2 and aurora1 share limited homology with each other and with the analogous 160 and 100 amino acid domains of *Drosophila* aurora and yeast Ipl1. However, the N-terminal regions of human and mouse aurora2 share 54% identity to each other and 28–30% identity to the two *Xenopus* proteins and together help define two distantly conserved motifs present in the non-catalytic region of all auroras (Figure 1A and B). These motifs are composed predominantly of conserved hydrophobic and polar residues. The first motif spans 18–37 amino acids (Aurora Box1), with aurora1 and yeast Ipl1 lacking the central portion and the second motif spans 21 amino acids (Aurora Box 2; see overlines in Figure 1A). Several potential serine and threonine phosphorylation sites are also conserved among these proteins, including a protein kinase A phosphorylation motif RRXT in the activation loop of the kinase. A temperature-sensitive mutant of the yeast *Ipl1* gene has a threonine to alanine substitution at this site (Francisco *et al.*, 1994), suggesting that phosphorylation on this threonine residue within the activation loop may be biologically relevant. Additional mutations in the yeast (Francisco *et al.*, 1994) and *Drosophila* (Glover *et al.*, 1995) homologues of aurora have been mapped exclusively to the kinase domain, except for a single *Drosophila* mutant (Glover *et al.*, 1995) that changes an aspartate to an alanine at residue 47 within the N-terminal Aurora Box1. Since these mutations result in abnormal nuclei, chromosome missegregation and monopolar spindles, these findings suggest that the catalytic activity of the auroras may play an important role in centrosome biology.

Aurora2 partially complements Ipl1

To determine whether human aurora1 and/or aurora2 are functionally equivalent to their *S.cerevisiae* homologue Ipl1, we attempted to complement the *ipl1-1* temperature-sensitive mutant strain, CCY464-1D (Francisco *et al.*, 1994) with expression plasmids encoding the aurora proteins. The CCY464-1D strain is inviable above 34°C due to a mutation in *Ipl1* (Francisco *et al.*, 1994). Neither aurora1 nor aurora2 was able to complement the *ipl1-1* mutation at 37°C, probably due to an inhibition of cell growth on overexpression of the unique N-terminal domains of these proteins (unpublished observation). To circumvent this problem, we generated expression plasmids, Ipl1/A1 and Ipl1/A2, containing the unique N-terminal domain of Ipl1 (amino acids 1–101), fused to the C-terminal catalytic domain of aurora1 (amino acids 75–344) or aurora2 (amino acids 131–403), respectively. Additional Ipl1/aurora fusions were made in which the essential lysine at residue 106 (K106) of aurora1 or residue 162 (K162) of aurora2 was mutated to a methionine resulting in catalytically inactive forms of both proteins. These kinase-dead constructs were designated Ipl1/A1KM and Ipl1/A2KM, respectively. These coding regions were subcloned into a CEN vector (Sikorski and Hieter, 1989) under control of the native *Ipl1* promoter. The wild-type Ipl1 construct complemented the *ipl1-1* mutation at 37°C, whereas no growth was observed with either the wild-type or kinase-dead fusions of Ipl1/aurora1 or Ipl1/aurora2 (Figure 2D). However, at the less restrictive temperature of 34°C, the Ipl1/A2 fusion partially complemented the *ipl1-1* mutation, whereas the kinase-dead Ipl1/A2KM and all aurora1 constructs failed to rescue the mutation (Figure 2C). Thus, in support of the conclusions derived from analysis of the primary amino acid sequence of these proteins, it appears that the aurora2 kinase is structurally and functionally equivalent to Ipl1, whereas aurora1 exhibits a biologically distinct activity.

Aurora1 and aurora2 are cell cycle regulated

Based on the predicted involvement of *Drosophila* aurora and yeast Ipl1 in centrosome separation and/or chromosome segregation, we investigated whether human aurora1 and aurora2 are cell cycle regulated. HeLa cells were synchronized at the G_1/S transition by a double thymidine/aphidicolin block (Golsteyn *et al.*, 1994) and followed through the completion of mitosis. After release from the G_1/S transition, the cells were analyzed for aurora1 and aurora2 RNA expression, protein expression and kinase activity. The DNA content at each time point was analyzed by flow cytometry (Figure 3A). Aurora1 and aurora2 RNA levels were low at the G_1/S transition (time = 0) and gradually increased as the cells progressed through S phase (time = 2–6 h) (Figure 3B, upper and middle panels) and through G_2 and mitosis (time = 8–10 h). Aurora1 RNA levels were highest at 8–10 h after release, corresponding to the G_2 and M phases of the cell cycle (Figure 3B, upper panel). The amount of aurora2 RNA peaked at 8 and 9 h post-release as the cells progressed from G_2 into mitosis and returned to low levels as the cells re-entered

G_1 at 12 h after release from the block (Figure 3B, middle panel). Actin RNA served as a loading control (Figure 3B, bottom panel). As expected, aurora1 and aurora2 proteins also varied during the cell cycle. Aurora1 and aurora2 proteins peaked at 8–11 h and 8–10 h after release, respectively (Figure 3C, upper and middle panels). $p34^{cdc2}$ protein levels served as a loading control (Figure 3C, bottom panel). We also examined aurora1 and aurora2 kinase activity during cell cycle progression. The aurora1 kinase activity was maximal during mitosis at 10–11 h after release (Figure 3D, top panel). Aurora2 kinase activity peaked at 9 h after release (Figure 3D, middle panel). $p34^{cdc2}$ kinase activity, which served as a marker for mitosis, peaked at 10 h after release (Figure 3D, bottom panel). Thus, both aurora1 and aurora2 RNA, protein and kinase activity were cell cycle-regulated, all being maximal during G_2 and mitosis. Aurora2 kinase activity was highest just prior to maximal activation of aurora1 and $p34^{cdc2}$. These data suggest that aurora2 function precedes that of aurora1 in mitosis.

Aurora1 and aurora2 are localized to mitotic structures

The subcellular location of endogenous aurora1 and aurora2 was determined by indirect immunofluoresence. Exponentially growing HeLa cells were fixed with methanol and probed with a monoclonal antibody to α-tubulin and with protein A affinity-purified antibodies to either aurora1 (Figure 3E, bottom panel) or aurora2 (Figure 3F, bottom panel). The aurora1 and aurora2 antibodies only stained structures in mitotic cells and did not stain any recognizable structures or compartments in interphase cells. This is understandable given that the proteins are most abundant at this stage of the cell cycle (Figure 3C). In anaphase and early telophase, the aurora1 antibodies stained the midzone and telophase disc (Andreassen *et al.*, 1991), whereas in late telophase and early G_1, they stained the post-mitotic bridge (Figure 3E, bottom panel). In metaphase and anaphase, the aurora2 antibodies stained the centrosome, spindle poles and the spindle (Figure 3F, bottom panel), whereas in telophase cells the aurora2 antibodies primarily stained the spindle pole (Figure 3F, bottom panel). The aurora2 immunostaining is consistent with that reported elsewhere (Gopalan *et al.*, 1997; Kimura *et al.*, 1997). The subcellular localization of aurora1 and aurora2 suggests that aurora1 may function later in mitosis than aurora2. This supports the observation that, in synchronized cells, aurora2 kinase activity precedes that of aurora1 (Figure 3D). In addition, the localization of aurora1 and aurora2 to mitotic structures confirms that they are indeed likely to be involved in chromosome segregation.

Expression of aurora1 and aurora2 RNA

Northern blot analysis of mRNA isolated from normal adult human tissues demonstrates that *aurora2* expression is primarily restricted to testis, thymus and fetal liver (Figure 4A), with very weak expression in bone marrow, lymph node and spleen, and no detectable expression in all other adult tissues examined. Human *aurora1* was also expressed at highest levels in normal thymus and fetal liver, with a moderate level of expression in lung and small intestine (Figure 4A).

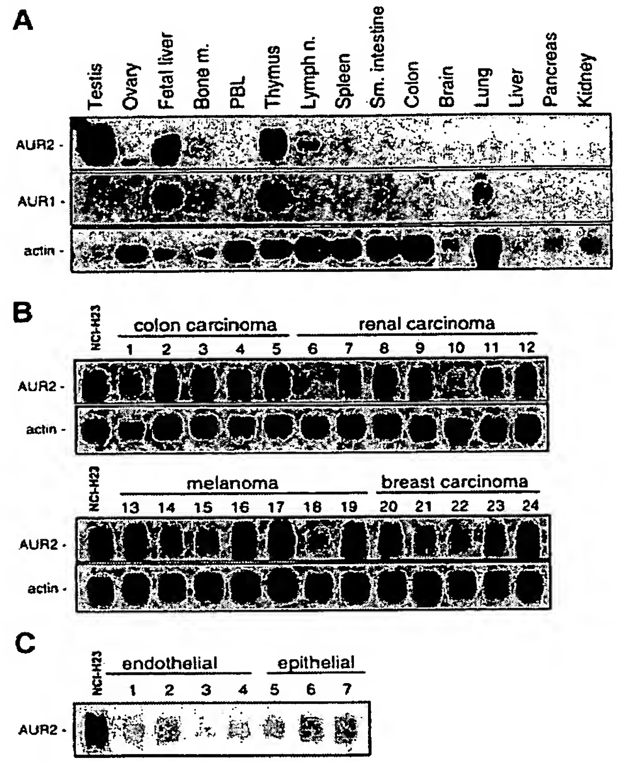

Fig. 4. Expression of human *aurora1* and *aurora2*. (A) Northern blot containing poly(A)$^+$ mRNA (2 μg per lane) from normal human tissue hybridized with an *aurora1* or *aurora2* DNA probe. (B) *Aurora2* Northern blots containing total RNA (20 μg) from human tumor cell lines. The single 2.4 kb *aurora2* transcript is marked. RNA from the lung cancer cell line NCI-H23 was included as a standard for the tumor blots. Cell lines included are: 1, HT-29; 2, HCC-2998; 3, COLO 205; 4, HCT-15; 5, KM012; 6, UO-31; 7, SN12C; 8, CAKI-1; 9, RXF393; 10, ACHN; 11, 786-0; 12, TK-10; 13, LOX IMVI; 14, SK-MEL-2; 15, SK-MEL-5; 16, SK-MEL-28; 17, UACC-62; 18, UACC-257; 19, M14; 20, MCF-7/ADR-RES; 21, HS 578T; 22, MDA-MB-435; 23, MDA-N; 24, T-47D. (C) *Aurora2* Northern blot containing total RNA (10 μg) from cultured primary human endothelial and epithelial cells. The single 2.4 kb *aurora2* transcript is marked. RNA from the lung cancer cell line NCI-H23 was included as a standard for the blots. Primary cell RNAs are: 1, coronary artery endothelial cells; 2, pulmonary artery endothelial cells; 3, lung microvascular endothelial cells; 4, dermal microvascular endothelial cells; 5, mammary epithelial cells; 6, renal proximal tubule epithelial cells; and 7, renal cortex epithelial cells.

Since aurora2 was highly represented in the initial PCR screen of primary colon tumors, we examined the expression of *aurora2* RNA in a panel of 25 human tumor cell lines of lung, colon, renal, melanoma and breast origin. The 2.4 kb *aurora2* transcript was expressed at high levels in 96% (24 of 25) of these transformed cell lines (Figure 4B), with the only exception being the UO-31 renal carcinoma cell line. Our preliminary analysis revealed that the 1.4 kb *aurora1* transcript was also expressed in the same 24 tumor cell lines (unpublished data), although we have yet to examine this in more detail. We also saw modest, but detectable, expression of *aurora2* in a panel of RNAs isolated from cultured primary epithelial and endothelial cells (Figure 4C). We conclude that *aurora2* is preferentially expressed in all rapidly

dividing cells, but its levels are significantly up-regulated in a broad range of tumor cell lines.

Amplification and overexpression of aurora2 in primary human colorectal cancers

The *aurora2* gene was mapped using the Stanford Human Genome Center G3 radiation hybrid panel. Human *aurora2* is located on chromosome 20q13.2 (LOD score of 17.26 to linked marker SHGC-3245). Mapping was also confirmed by hybridization to a human–rodent somatic cell hybrid panel (Coriell Cell Repository, Camden, NJ). *Aurora2* maps adjacent to the vitamin D hydroxylase (*CYP24*) gene and the cosmid probe RMC20C001 that lie at 0.825–0.83 Flpter (fractional length from pter) on chromosome 20 (Tanner *et al.*, 1994, 1996). Both of these markers have been characterized for their presence in the 20q13 amplicon common to many human malignancies, particularly those from breast, bladder and colon cancers (Muleris *et al.*, 1987; Bigner *et al.*, 1988; Yaseen *et al.*, 1990; Kallioniemi *et al.*, 1994; Tanner *et al.*, 1994, 1996; Iwabuchi *et al.*, 1995; Schlegel *et al.*, 1995; Bockmuhl *et al.*, 1996; Courjal *et al.*, 1996; Reznikoff *et al.*, 1996; Solinas-Toldo *et al.*, 1996; James *et al.*, 1997; Larramendy *et al.*, 1997).

Since the *aurora2* gene maps to a prevalent tumor amplicon, we questioned whether the *aurora2* gene was also amplified in a cohort of primary human colorectal tumors and matched normal colorectal tissue from the same patients. Southern blot hybridization was performed using an *aurora2* cDNA probe along with a control probe for the *CYP24* gene that serves as a marker of the amplicon (Tanner *et al.*, 1994, 1996). The *aurora2* probe hybridized to *Pst*I fragments of 5.8, 3.7, 3.3, 2.8, 2.5 and 1.3 kb. The 5.8, 3.3, 2.8 and 2.5 kb bands are specific to *aurora2*, while the 3.7 and 1.3 kb bands represent cross-hybridization to the *aurora3* and *aurora4* pseudogenes which map to chromosomes 1 and 10, respectively. Only the *aurora2*-specific bands showed amplification in the tumor samples. *Aurora2* DNA was amplified in (52%) 41 of 79 of the primary colorectal tumors for which suitable DNA was available for genotyping (Figure 5B). The *CYP24* gene was found to be co-amplified with *aurora2* in (90%) 37 of 41 matched pairs and was found only once to be amplified in the absence of *aurora2* amplification.

Aurora2 RNA levels were characterized by Northern blot analysis of samples from the same panel of matched tumor/normal tissues. Approximately 54% (22 of 41) of the tumors showed increased expression of the 2.4 kb *aurora2* transcript as compared with the normal colon control. *Aurora2* RNA showed 4- to 28-fold overexpression in tumor versus normal tissue. Representative Northern and Southern data from 12 matched tumor/normal pairs are shown in Figure 5, where nine samples demonstrated amplification of *aurora2* DNA in the range of 2- to 8-fold in the tumors compared with normal tissue (2164, 2172, 2193, 3204, 2255, 3189, 3191, 3193 and 2176) and three samples (1985, 2175 and 2257) showed no amplification. This level of *aurora2* amplification is consistent with other reports of 1.5- to 10-fold increases in copy number of 20q13 in primary tumors and tumor cell lines (Kallioniemi *et al.*, 1994; Tanner *et al.*, 1996; Sen *et al.*, 1997). Sample 3193 still shows a relative level of DNA amplification after adjusting for unequal sample loading. One of the samples (1985) clearly demonstrates RNA overexpression in the absence of DNA amplification, whereas the other 11 show a direct correlation between DNA amplification and RNA overexpression. We obtained complete data for analysis from 37 matched sets of RNA and DNA from both normal and tumor samples. Data in Table I show a high correlation ($\rho = 0.695$, Pearson correlation; $P < 0.00003$, Fisher's exact test) between *aurora2* DNA amplification and RNA overexpression with only one discordant result. In the single case of *aurora2* DNA amplification in the absence of RNA overexpression, *aurora2* RNA was actually elevated in both the normal and tumor specimens, compared with other tumor/normal pairs. It is conceivable that high expression of *aurora2* RNA in this normal colon sample may represent an early predisposing lesion. Conversely, five paired samples showed increased RNA expression in the absence of DNA amplification, possibly due to transcriptional activation. If these five pairs are excluded from the analysis, the correlation between *aurora2* DNA amplification and RNA overexpression increases to $\rho = 0.939$. These data suggest that DNA amplification is a mechanism for *aurora2* activation and also implicates *aurora2* as an oncogene at 20q13 whose high level amplification correlates with poor clinical outcome in breast cancer (Isola *et al.*, 1995).

To determine if the *aurora2* sequence from the 20q13 amplicon was the same as that from normal sources, we performed direct sequencing of RT–PCR products encompassing the complete *aurora2* coding region from 10 primary colorectal tumor samples. Eight samples, including tissues with both normal and amplified levels of the 20q13 amplicon, confirmed the *aurora2* sequence. A single base change was identified in two samples (1985 and 2193) resulting in a phenylalanine to isoleucine change at residue 31 in the N-terminal Aurora Box1 (circled in Figure 1A). Experiments are planned to determine if this is simply a polymorphism or whether this change affects aurora2 activity. Nonetheless, these analyses demonstrate that the 20q13 amplicon typically contains increased copies of the intact, unmutated *aurora2* coding region.

Detection of aurora2 protein in primary human colon cancer samples

To determine whether the amplification of *aurora2* gene and message resulted in overexpression of the protein, anti-aurora2 antibodies were used to probe blots of protein lysates made from cryostat sections of primary human colon carcinomas or from adjacent normal tissue isolated from the same patient. As shown in Figure 6A, the aurora2 antibodies detected a protein of ~46 kDa in the two primary human colon carcinomas, but not in samples derived from the adjacent normal tissue. Due to the limited amount of tissue available, we were unable to determine if *aurora2* was amplified in these samples. These antibodies also detected overexpression of aurora2 protein in various cultured tumor cell lines derived primarily from colorectal carcinomas (Figure 6B). While most tumor cell lines examined expressed detectable levels of aurora2 protein, others including A549 cells do not (Figure 6B, lane 1).

Aurora2 transforms Rat1 fibroblasts

If *aurora2* is a relevant target on the 20q13 amplicon, one might expect that overexpression of *aurora2* would be

Tumour amplified kinase *STK15/BTAK* induces centrosome amplification, aneuploidy and transformation

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The centrosomes are thought to maintain genomic stability through the establishment of bipolar spindles during cell division, ensuring equal segregation of replicated chromosomes to two daughter cells. Deregulated duplication and distribution of centrosomes have been implicated in chromosome segregation abnormalities, leading to aneuploidy seen in many cancer cell types. Here, we report that *STK15* (also known as *BTAK* and *aurora2*), encoding a centrosome-associated kinase, is amplified and overexpressed in multiple human tumour cell types, and is involved in the induction of centrosome duplication-distribution abnormalities and aneuploidy in mammalian cells. *STK15* amplification has been previously detected in breast tumour cell lines[1] and in colon tumours[2]; here, we report its amplification in approximately 12% of primary breast tumours, as well as in breast, ovarian, colon, prostate, neuroblastoma and cervical cancer cell lines. Additionally, high expression of *STK15* mRNA was detected in tumour cell lines without evidence of gene amplification. Ectopic expression of *STK15* in mouse NIH 3T3 cells led to the appearance of abnormal centrosome number (amplification) and transformation *in vitro*. Finally, overexpression of *STK15* in near diploid human breast epithelial cells revealed similar centrosome abnormality, as well as induction of aneuploidy. These findings suggest that *STK15* is a critical kinase-encoding gene, whose overexpression leads to centrosome amplification, chromosomal instability and transformation in mammalian cells.

Amplification of chromosome 20q DNA has been reported in breast[3], bladder[4], chondrosarcomas[5], colon[6], ovarian[7], pancreatic[8] and head and neck cancers[9]. DNA amplification on chromosome 20q13 has also been correlated with poor prognosis among axillary node negative breast tumour cases[10]. Earlier, we reported cloning of a partial cDNA encoding *STK15* from this amplicon, and found that it is amplified and overexpressed in three human breast cancer cell lines[1]. To determine whether *STK15* is the relevant target gene in this amplicon, we investigated *STK15* amplification/expression in a variety of tumour cell lines and primary breast tumours, in addition to characterizing its product and function in mammalian cells.

Southern-blot hybridization revealed 2.5–8-fold amplification of *STK15* in cell lines (Fig. 1*a*). We found *STK15* to be highly expressed in the majority of the tumour cell lines with gene amplification (Fig. 1*b*); high expression of *STK15* mRNA in the absence of increased gene copy number was also observed. We verified that the gene copy increments in tumour cells did not result from polysomy by fluorescence *in situ* hybridization (FISH), using a gene-specific probe and centromeric α-satellite or 'p' arm-specific (for chromosome 20) probes. The *STK15* probe localized to chromosome 20q13 (Fig. 2*a*,*b*). Co-hybridization of *STK15* and 'p' arm probes on normal breast epithelial cells generated two signals for each probe, as expected (Fig. 2*c*). Amplified signals on multiple chromosomes revealed intra-chromosomal amplification of *STK15* in BT474 cells (Fig. 2*d*). Multiple signals for the *STK15* probe, in parallel with two signals for the chromosome 20 'p' arm probe (Fig. 2*e*,*f*), documented amplification of the gene in tumour tissues. Assuming that six or more signals are indicative of amplification, we detected *STK15* amplification in approximately 12% of primary breast tumours.

The full-length *STK15* cDNA sequence revealed an ORF encoding a 403-aa protein with a molecular weight of approximately 46 kD. The amino acid sequence revealed conservation of the 12 kinase-specific subdomains[11]. *STK15* showed approximately 48% and 40% overall sequence identity with the *Drosophila melanogaster* and *Saccharomyces cerevisae* serine/threonine kinases, *aurora* and *Ipl1*, respectively[12,13]. A human kinase (*Aik*) with high homology to *STK15* has also been reported[14], as have the mouse orthologues of *STK15*, *Ayk1* (ref. 15) and *Iak1* (ref. 16).

Protein expression analyses carried out with affinity-purified anti-*STK15* antipeptide antibodies revealed a prominent *STK15* polypeptide in BT474 cells (Fig. 3*a*). These antibodies also quantitatively immobilized STK15 (Fig. 3*b*). The STK15 immunocomplex from BT474 cell lysate revealed high levels of casein and MBP phosphorylating activities (Fig. 3*c*), indicating that STK15 is a functional kinase. Like cellular STK15, His-tagged recombinant STK15 also has high levels of casein kinase activity (Fig. 3*d*).

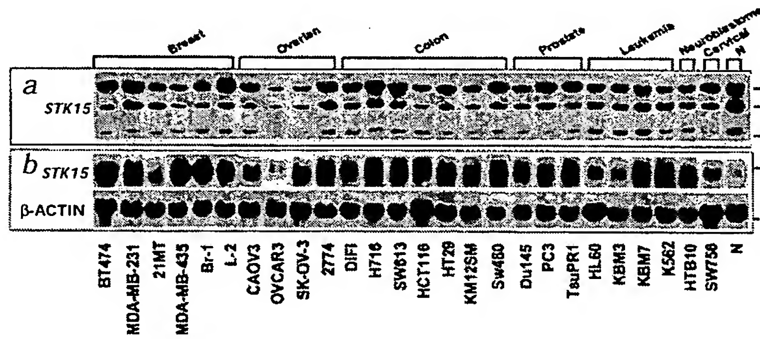

Fig. 1 Southern- and northern-blot hybridization analyses estimating *STK15* copy number (*a*) and *STK15* mRNA expression (*b*) in tumour cell lines. The top band of approximately 13.5 kb in the *Bam*HI digests, representing *STK15*-specific sequences, and the third band of approximately 7.9 kb, representing unamplified sequences, were included in signal intensity measurements to estimate gene copy number. Lane N contained human lymphocyte DNA from a normal healthy individual. The names of cell lines from which DNA were derived are indicated. The highest level of amplification (8×) was seen in the breast tumour cell lines BT474 and MDA-MB-231, whereas approximately 2.5× to 5× amplification was seen in ovarian (2774), colon (DiFi, H716, KM12SM, SW480), prostate (PC3, TsuPR1), neuroblastoma (HTB10) and cervical (SW756) tumour cell lines.

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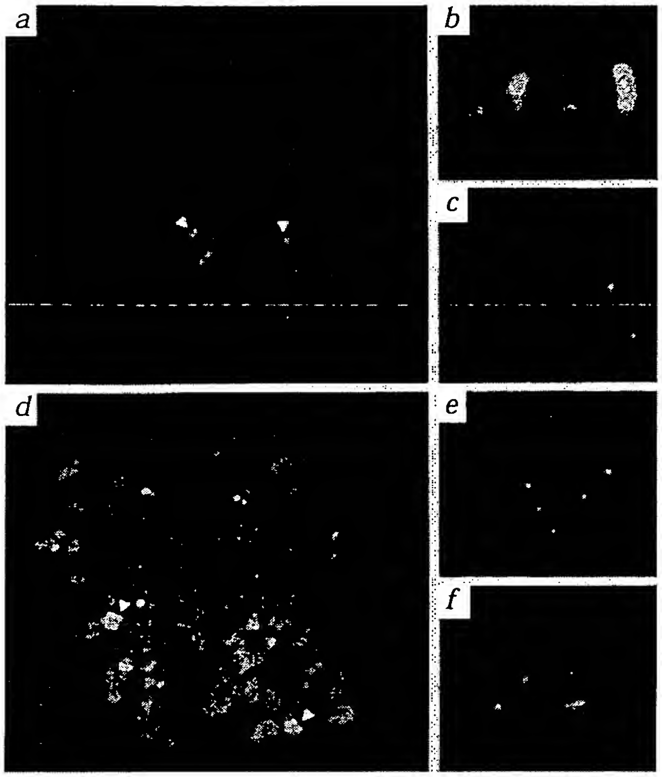


Fig. 2 Dual colour FISH demonstrating localization of the *STK15* probe (green signals) along with chromosome-20–specific centromeric probe or a 'p' arm-specific probe (red signals) on normal human chromosome 20 (***a,b***) and diploid copy number of *STK15* in the interphase nucleus of normal breast epithelial tissue (***c***). Amplification of *STK15* was detected by single colour FISH using FITC-labelled *STK15* probe (yellow signals) on chromosomes of human breast cancer cell line BT474 (***d***) and dual colour FISH using FITC-labelled *STK15* probe (green signals) along with Cy3-labelled chromosome 20 'p' arm probe (red signals) in interphase nuclei of primary human breast tumour tissues (***e,f***). Note multiple *STK15* signals in contrast with two signals for the 'p' arm probe in the nuclei.

To investigate STK15 expression and kinase activity through the cell cycle, protein lysates prepared from exponentially growing and synchronized mitotic cells were analysed by immunoblot and immunocomplex kinase assays. We observed a 46-kD band on blotting mitotic lysate samples (Fig. 4*a*), and the 'CDC2' band in both random population and mitotic cell lysates (Fig. 4*b*). The STK15 protein (Fig. 4*c*) and casein kinase activity (Fig. 4*d*) were also detectable at higher level in the immunocomplex from mitotic cell lysates.

Indirect immunofluorescence microscopy of HeLa cells demonstrated that STK15 is localized on centrosomes in interphase cells and at each spindle pole in mitosis (Fig. 5*a*). Similar centrosome localization in mitosis for *Aik* (ref. 14) and *aurora 2* (ref. 2) have been described. To determine the effect of elevated *STK15* expression on centrosomes, we transiently transfected NIH 3T3 cells with *STK15* expression constructs. The majority of vector-transfected cells revealed two centrosomes, but a significant proportion (approximately 22%) of *STK15*-transfected cells revealed multiple sites of STK15 localization, which correlated with amplified centrosomes (Fig. 5*b,c*). We also characterized these transfectants with respect to *in vitro* growth and their potential for transformation. Three clones with each construct were grown in different concentrations of serum (0.5% and 5% bovine calf serum) and in soft agar. Vector-transfected cells failed to grow as monolayers in 0.5% serum (Fig. 5*d*, left) and mostly formed clusters of 3–4 cells in soft agar (Fig. 5*d*, right). It was evident that these cells do not form large colonies in soft agar, even if grown for longer periods of time. *STK15*-transfected cells grew to confluence as monolayers, forming foci under identical conditions (Fig. 5*e*, left), and grew in soft agar as discrete colonies (Fig. 5*e*, right). The average number of colonies per 2,000 cells plated from each transfection were 447 for cells stably transfected with *STK15* and 32 for those transfected with vector alone. Three independent experiments with cells stably transfected with *STK15* showed that these cells maintained a transformed phenotype only in 0.5%, and not in 5%, serum-containing media. *STK15*-transfected human breast carcinoma MCF7 cells also display similarly altered growth characteristics in reduced serum. It is likely that in low serum, the minimal presence of certain factor(s) allows promiscuous activity of STK15 protein, leading to transformation of NIH 3T3 cells. This might explain why in a recent publication, *aurora2*, which is identical to *STK15*, was reported to lack the capacity to transform NIH-3T3 cells[2]. Transformation of Rat-1 fibroblasts by *aurora2* described in this publication suggests that the serum-inducible signalling cascade influencing STK15 activity may be altered in Rat-1 fibroblasts.

We hypothesized that overexpression of *STK15* causes centrosome amplification and aberrations in chromosome partitioning at mitosis, leading to catastrophic loss or gain of chromosomes and resulting in either cell death or survival through malignant transformation. To test this hypothesis, we transiently transfected a *STK15* expression construct into near diploid human breast epithelial cell line MCF10A (ref. 17), which has normal

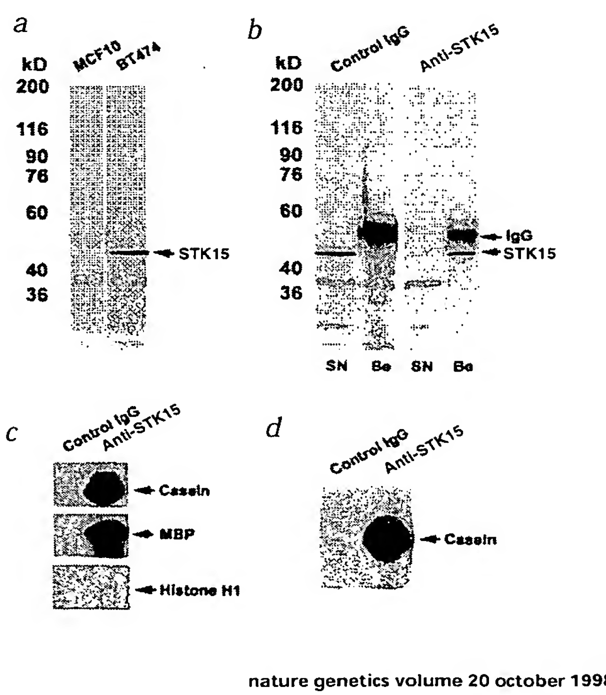


Fig. 3 Expression of STK15 and kinase activity. Cell lysates were made from exponentially growing MCF10 or BT474 cells. ***a***, Note high expression of STK15 in BT474 cell lysate immunoblotted with affinity-purified anti-STK15 antibodies. ***b***, Protein extract of BT474 cell lysate absorbed with either anti-STK15 antibody-protein G agarose or preimmune serum IgG-protein G agarose. Proteins in the supernatant (SN) and on the beads (Be) were immunoblotted with anti-STK15 antibodies. Note selective immobilization of STK15 by anti-STK15 antibodies. ***c***, Proteins from BT474 cell lysates were immunoadsorbed with either control antibody- or anti-STK15 antibody-affinity beads to do immunecomplex kinase assay with casein, MBP or histone H1 in the presence of [γ^{32}P]ATP. Note, STK15 immunocomplex from BT474 cells exhibited high levels of casein and MBP phosphorylating activities. ***d***, Purified recombinant STK15, absorbed with anti-STK15 antibody-affinity beads, also revealed casein kinase activity.

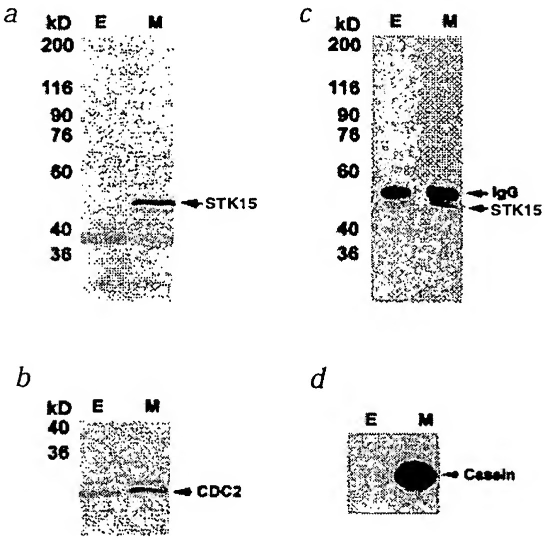
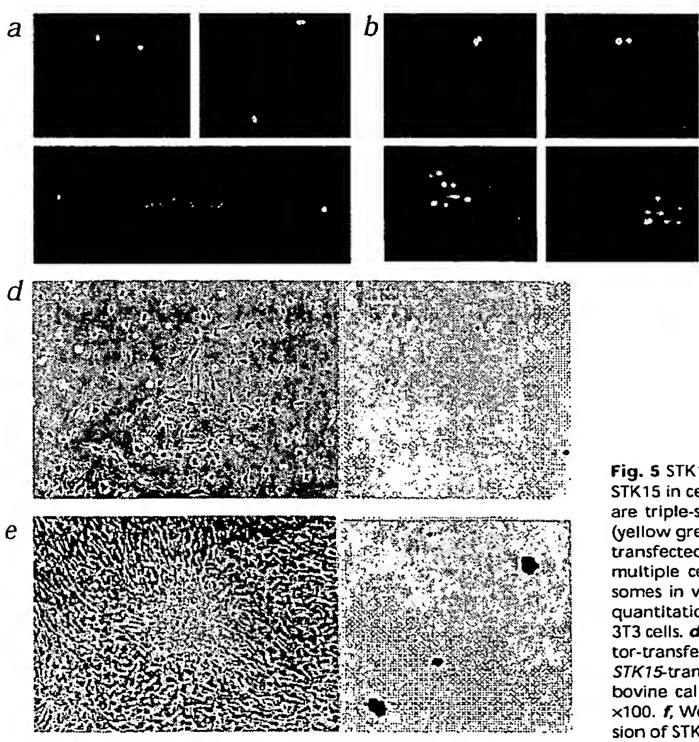


Fig. 4 Cell lysates from exponentially growing (lane E) or colcemid synchronized mitotic (lane M) HeLa cells were prepared and proteins (50 μg) from each lysate were separated by 12.5% SDS-PAGE and transblotted onto nitrocellulose. The blots were stained with affinity-purified anti-STK15 antibodies (*a*) and with affinity-purified anti-CDC2 antibodies (*b*). Similar amounts of cell lysate were absorbed with anti-STK15 antibody-affinity beads. Proteins on the beads were immunoblotted with anti-STK15 antibodies (*c*) and assayed for casein kinase activity (*d*).

disomy for chromosomes 13, 21 and X. We performed ploidy analyses of these chromosomes with centromeric α-satellite DNA probes. Assuming normal chromosome segregation, these cells would reveal in most instances six signals. Deviations from this number would indicate abnormal chromosome segregation and induction of aneuploidy. A significant number of cells transfected with *STK15* expression construct revealed signals either less or more than six, unlike the vector-transfected cells which predominantly displayed six signals (Fig. 6*a–c*). We also observed aberrant chromosome segregation in dividing *STK15*-transfected cells (Fig. 6*d,e*) and a significant increase in aneuploidy (Fig. 6*f*) among *STK15* transfectants (approximately 18% of these had less than six centromeres; and approximately 8% had over six centromeres), compared with vector-transfected cells (approximately 2% with less than six centromeres; approximately 4% with over six centromeres). Approximately 12% of *STK15* transfectants revealed more than two centrosomes, compared with less than 3% of the vector-transfected cells showing a similar phenotype (Fig. 6*g*).

Phosphorylation of centrosomal proteins in *Drosophila*[18] and vertebrates[19–21] influence microtubule nucleation and dynamics at the centrosomes[22]. In mammalian cells, NIMA, CDC2 and PLK1 kinases have been implicated in centrosome duplication, maturation and separation[23–25]. Overexpression of PLK1 has recently been associated with non-small cell lung cancer[26], and was reported to transform NIH 3T3 cells[27] although convincing evidence demonstrating that aberrations in these proteins are responsible for malignant transformation in human cells as a consequence of centrosome aberrations has yet to be presented. Recently, centrosome duplication abnormalities have been correlated with both genomic instability and the deletion or mutation of *TP53* (refs 29,30) and study of 35 high-grade human breast tumours[31] suggested that structural and functional centrosome defects have implications for understanding genomic instability in solid tumours.

Identification of mutations in the mitotic checkpoint gene *BUB1* in human cancers[32] represented the beginnings of a proof that acquired aneuploidy may be a specific driving force in tumour progression, rather than an epiphenomenon of this disease[33]. A report published last month implicates *MET* in mediating non-random chromosome duplication (of the chromosome carrying mutant allele; ref. 34). Together with these studies, our



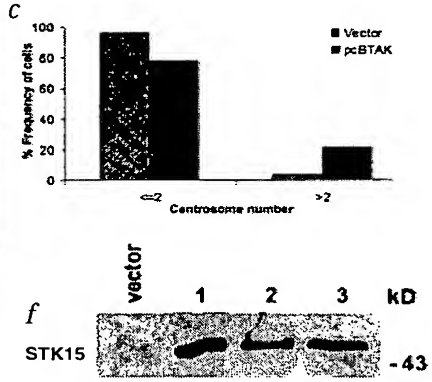


Fig. 5 STK15 expression and centrosomes in mammalian cells ***a***, Localization of STK15 in centrosomes of HeLa cells at prophase, metaphase and telophase. Cells are triple-stained with DAPI for DNA (blue), anti-tubulin (red) and anti-STK15 (yellow green). ***b***, Localization of STK15 (left) and centrosomes (right) in vector-transfected (top row) and *STK15*-transfected (bottom row) NIH3T3 cells. Note multiple centrosomes in *STK15*-transfected cells in contrast with two centrosomes in vector-transfected cells. Cells are triple-stained as described in (*a*). ***c***, quantitation of centrosome numbers in 200 vector and *STK15*-transfected NIH 3T3 cells. ***d,e***, Monolayer (left) and soft agar (right) growth pattern of stable vector-transfected (*d*) and *STK15*-transfected (*e*) NIH 3T3 cells. Focus formation by *STK15*-transfected cells was observed in monolayer cultures grown with 0.5% bovine calf serum. Photomicrographs were taken at a total magnification of ×100. ***f***, Western-blot analyses of *STK15*-transfected 3T3 clones showing expression of STK15.

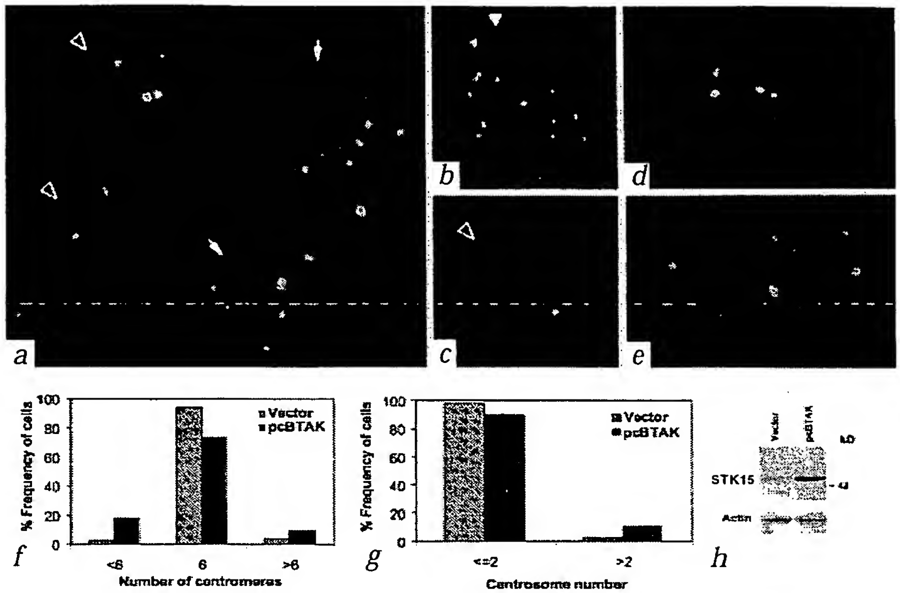


Fig. 6 FISH localization of centromeres of chromosomes 13, 21 and X in *STK15*-transfected near-diploid breast epithelial cell line MCF10A. ***a–c***, nuclei with less than six (open arrowheads), equivalent to six (arrows) and exceeding six (filled arrowhead) centromeres. ***d,e*** unequal segregation of centromeres in dividing cells. ***f***, Centromere signal number variations quantitated from 200 vector and *STK15*-transfected MCF10A cells. ***g***, Centrosome number variations quantitated in 200 vector and *STK15*-transfected MCF10A cells. ***h***, Western-blot analyses of vector- and *STK15*-transfected cells showing *STK15* expression in the transfectants. The same blot was probed with actin antibody to verify equal amount of protein loading in the two lanes.

finding that STK15 overexpression induces aneuploidy in mammalian cells suggests that specific gene abnormalities can directly influence chromosome ploidy in tumour cells. Identification of the natural substrates of STK15 will help to elucidate its biochemical pathway, the disruption of which would appear to result in anomalous centrosome amplification and chromosome segregation in tumour cells. These may, in turn, lead to new genetic targets and appropriate drugs for the therapy of tumours with chromosomal instability.

Methods

cDNA cloning and sequencing. To isolate the complete *STK15* cDNA, we screened a BT474 cDNA library, constructed with cDNA synthesis kit (Superscript choice system from GIBCO/BRL) in λ-Zap II vector (Stratagene) with the insert from the partial cDNA clone pcBTAK$_7$ (ref. 1). We isolated and sequenced overlapping clones with the help of Applied Biosystems Model 373A Automated DNA sequencer, available at the core sequencing facility of University of Texas M.D. Anderson Cancer Center. We sequenced both strands and identified the 5′ end of the mRNA by 5′-RACE, with the help of the 5′-RACE system for rapid amplification of cDNA ends, Version 2.0 kit (GIBCO/BRL), according to the manufacturer's instructions.

Isolation of genomic BAC clones and FISH analysis. We screened human genomic BAC libraries from California Institute of Technology (Research Genetics) by hybridization with pcBTAK$_7$ cDNA probe. For FISH analyses of tumour tissues, we co-hybridized a *STK15*-specific BAC probe with either a chromosome-20–specific centromeric α-satellite probe or with a chromosome-20, short-arm–specific, *D20S894* probe. Other genomic probes (200 ng each) were labelled with FITC or Cy3 by using Prime-it Fluor fluorescence labelling kit (Stratagene) or by nick translation using the Nick translation kit (Gibco/BRL). Of the 84 tumours analysed, 45 were paraffin-embedded histological sections and 39 were 'snap'-frozen touch preps from unselected primary breast tumour patients. Slides were digested with pepsin (40–100 μg/ml) in 0.01 N HCl at 37 °C and denatured in 70% formamide, 2×SSC, pH 7.0 at 72 °C. An aliquot (10 μl) of probe mixture containing labelled probe (200 ng), human Cot-1 DNA (10 μg) in 55% formamide, 10% dextran sulfate, 1×SSC was denatured and applied on pretreated slides. We incubated slides at 37 °C for 2–3 d, washed them in 4×SSC, 0.1% Triton X-100 at 37 °C, rinsed them in distilled water and then laminated them with DAPI/antifade or PI/antifade. For chromosome ploidy analyses, we used digoxigenin-labelled, centromeric α-satellite probes for chromosomes 13, 21 and X (Oncor), according to the manufacturer's instructions. Slides were viewed with Zeiss and Nikon fluorescent microscopes.

Immunoprecipitation, immunoblotting and immunecomplex kinase assay. To prepare protein extract of cell lysates, we lysed them by sonication with five volumes of extraction buffer (80 mM Na β-glycerophosphate, 20 mM EDTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 μM okadaic acid) and protease inhibitor (10 μg/ml of each; leupeptin, pepstatin A and chymostatin; Boehringer). We determined total protein concentrations by Bradford analysis. A polyclonal anti-STK15 antibody raised against a carboxy-terminal peptide in rabbit was affinity purified. For immunoprecipitation, protein extract (50 μg) of cell lysates were absorbed with 10 μl of anti-STK15 antibody-protein G agarose or preimmune serum IgG-protein G agarose. Proteins in the supernatant and on the beads were sparated on 12.5% SDS-PAGE and then immunoblotted with anti-STK15 antibodies. For immune-complex kinase assay, we immobilized affinity-purified anti-STK15 antibodies on Affi-prep protein A matrix (Bio-Rad) at a concentration of 0.1 mg/ml, incubated them with 10 volumes of BT474 cell lysates at 4 °C for 4 h. The pelleted beads were washed with Tris HCl (100mM, pH 7.3), NaCl (150 mM), DTT (1 mM) and NP-40 (0.5%). We assayed the ability of STK15 to phosphorylate α casein (Sigma), myelin basic protein (Sigma) or histone H1 (Boehringer) using immunocomplex reactions in the presence of the substrates, incubated with 0.5 μci/μl [λ-P32] ATP at RT for 30 min and stopped them by addition of SDS-PAGE sample buffer. We separated proteins using 12.5% SDS-PAGE and the dried gels were subjected to autoradiography.

Expression construct and transfection. We cloned *STK15* cDNA (1.8 kb) at the *Bam*HI and *Xho*I sites of the mammalian expression vector pcDNA 3.1 (Invitrogen) for high level expression under the control of cytomegalovirus enhancer promoter. For stable transfection, we added DNA (1 μg) mixed with lipofectamine reagent (12 μl; Gibco BRL) to 3×10^5 cells in a 60-mm dish. After 5 h incubation in serum-free medium, we added complete medium with serum to the cells and incubated them for 48 h. We selected stable clones with 600 μg/ml G418. For 3T3 focus formation assay, 1×10^6 cells from stable clones were grown in a 100-mm dish in medium containing bovine calf serum. *STK15* stable transfectants formed foci after about 10 d. For transient transfection, we added DNA lipofectamine mix to 3×10^5 cells and incubated them in serum-free medium for approximately 12 h. We then added fresh complete medium, and harvested cells after 48 h for immunofluorescence staining and immunoblotting analyses.

Soft agar assay. We supplemented agar (0.35%, 4ml) in Dulbecco's Modified Eagle Medium (DMEM) with 5% bovine calf serum and poured it into a 60-mm dish to form bottom agar. We layered agar mix (0.35%, 1 ml) with 2,000 cells on top, and incubated the dishes at 37 °C in a 5% CO_2 atmosphere. We added fresh top agar (0.3 ml) each week; after 3 weeks, we stained the plates overnight with p-iodonitrotetrazolium violet (Sigma) at 37 °C.

Immunofluorescence microscopy. For immunofluorescence staining, we grew cell monolayers to confluence on poly-D-lysine coated glass coverslips and permeabilized in 0.5% TritonX-100 in PEM+PEG buffer (80 mM PIPES, pH 7.0, 5 mM EGTA, 2 mM $MgCl_2$, 4% polyethylene glycol) at RT. Cells were fixed in 3.7% paraformaldehyde in PEM+PEG buffer at RT. Subsequently, the coverslips were washed in buffer, treated with 5% normal goat serum and incubated with a 1:1 mixture of anti STK15 antibody (diluted 1:200) plus TU27B anti-tubulin antibody (courtesy of L.I. Binder) at 37 °C for 1 h. For immunostaining of centrosomes, we used two rabbit antisera against γ tubulin and auto-antibodies against centrosomes. The cells were washed in buffer and exposed to secondary antibodies consisting of a mixture of FITC-conjugated goat anti rabbit antibody (Pierce) and Texas Red-conjugated goat anti-mouse antibody (Molecular Probes) for 1 h at 37 °C. We washed coverslips in buffer, counterstained with DAPI and attached them to glass slides with antifade mounting media (Vectashield). All images were collected on a Zeiss Axiophot fluorescence microscope equipped with Hamamatsu high resolution/sensitivity CCD video camera and digitally processed using Adobe PhotoShop.

Southern- and northern-blot analysis. Southern- and northern-blot procedures were performed according to standard protocol[1]. We used DNA (10 μg) digested with *Bam*HI for Southern-blot analysis. Signal intensities for the top and the third bands on Southern blots were measured by Phospohorimager analyses using a Model GS-363 system (Bio-Rad). *STK15* copy number estimations in the tumour cells were done in reference to the normal diploid amount estimated in lymphocytes. We used total RNA (10 μg) for northern-blot analysis. The same blot was hybridized with a β actin probe to normalize the amount of RNA loading in each lane.

GenBank accession number. *STK15* cDNA, AF011468.

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Exhibit D

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Advances in Brief

Centrosomal Kinase AIK1 Is Overexpressed in Invasive Ductal Carcinoma of the Breast[1]

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Abstract

A centrosomal serine/threonine kinase, AIK1[3]/ breast tumor amplified kinase/aurora2, which was recently identified as an oncogene, shows high amino acid identity with chromosome segregation kinases, fly Aurora, and yeast Ipl1. Immunohistochemical analyses of invasive ductal adenocarcinomas of the breast revealed that overexpression of AIK1 was observed in 94% of the cases, irrespective of the histopathological type, whereas the protein was not detected in normal ductal and lobular cells. Benign breast lesions including fibrocystic disease and fibroadenoma (epithelial components) displayed weakly detectable AIK1 expression in part of the lesions. This is the first immunohistochemical report of AIK1 expression in primary human breast carcinomas. Although the physiological function(s) of AIK1 kinase during cell division remains to be determined, the markedly high positivity of AIK1 staining in the cancer lesions suggested a possible involvement of its overexpression in the tumorigenesis of some of breast cancer cells.

Introduction

Cancer is a genetic disease resulting from an accumulation of genetic abnormalities in various cell cycle-regulatory genes (1). A multistep genetic model of tumorigenesis has been proposed for neoplasms such as colon cancers (2). Mutability is acquired in most tumors as they progress. Studies on early colorectal cancer have suggested that genetic instability is a prominent feature of preinvasive cancer (3). During the evolution of normal cells into cancer cells, the occurrence of multiple mutations results in genetic instability. A variety of chromosome aberrations, such as abnormal ploidy, are common in cancer cells (4–9). The centrosome is believed to play a unique role in maintaining genomic stability by establishing bipolar spindles during cell division. Equal segregation of duplicated chromosomes into two daughter cells is ensured through the actions of tightly regulated centrosome function. Centrosome amplification is often observed in cancer cells, and this abnormality is thought to cause chromosomal missegregation, which is important for the progression of malignancy (10).

Yeast *IPL1* and fly *aurora* gene products are known to constitute a family of serine/threonine kinases that are involved in normal chromosome segregation (11, 12). Loss or dysfunction of *aurora* causes a failure of the centrosomes to separate and form a bipolar spindle (12). Conditional *ipl1*^{ts} mutants missegregate chromosomes, leading to an increase in ploidy (13). Although the substrate(s) and the regulator(s) of these kinases have not been identified, type 1 protein phosphatase acts in opposition to Ipl1 protein kinase in yeast (11). Recent studies by our group and other investigators revealed the presence of the following members of the Aurora/Ipl1-related protein kinase family in vertebrates: (*a*) human AIK1/BTAK[3, 4]/aurora2/ARK1 (14–17); (*b*) mouse STK1 (18) and Ayk1/IAK1 (19, 20); (*c*) rat AIM-1 (21); (*d*) human ARK2/AIK2 (17, 22); and (*e*) *Xenopus* pEg2 (23), all of which have highly related COOH-terminal kinase domains. The similarity of the NH_2-terminal regulatory domains indicated that these fall into two subgroups: (*a*) human AIK1, mouse Ayk1/IAK1, and *Xenopus* pEg2 constitute the AIK1 subfamily; and (*b*) human AIK2, mouse STK1, and rat AIM-1 constitute the AIK2 subfamily. Furthermore, recent investigations by us and others have revealed the presence of a third subgroup, AIK3/STK13 (24, 25). AIK1 (14, 16) and AIK3 (24) localize at centrosome, and AIM-1 and AIK2 localize at the midbody (17, 21). Although the biological functions of these kinases are not yet well understood, the overexpression of AIK1 in fibroblasts induced centrosome amplification (26), and dominant negative AIM-1 caused the failure of cytokinesis, which resulted in cell cycle arrest and multinucleation (21).

Previous investigations revealed chromosome aberrations at chromosome 20q13 in cancer tissues of several organs (27–31), and increased copy numbers at 20q13 were frequently observed in low-grade ovarian tumors (28). Studies using comparative genomic hybridization indicated that a major locus for DNA amplification in breast cancer is located at 20q13 (32). The gene for *AIK1/BTAK* (approved gene symbol is *STK6/STK15*) was mapped to human chromosome 20q13.2–13.3 (15, 33). In addition, the *BTAK/aurora2* gene is amplified, and its protein product is overexpressed in breast and colorectal cancer cell lines (15, 16). Because AIK1 has a high amino acid identity with Aurora and Ipl1, which play a role in chromosome segregation, its abnormalities may affect certain oncogenic processes. AIK1 protein has been shown to localize to the spindle pole during mitosis, especially from prophase through anaphase, suggesting a possible involvement of AIK1 in some centrosome functions (14). Because the protein has been suggested to regulate some centrosomal function(s), a defect in its regulation might cause an alteration in the chromosome number. In fact, recent studies revealed that overexpression of human BTAK/AIK1 in rodent fibroblasts induced centrosome amplification, aneuploidy, and transformation, indicating that BTAK/AIK1 is oncogenic (16, 26).

In the present study, we examined the expression of AIK1 protein in invasive ductal carcinomas of the breast with various histopathological types to highlight its significance in the pathogenesis and/or prognosis of human cancers. Immunohistochemical analyses showed a strong AIK1 staining in the majority of cancers, suggesting a possible involvement of AIK1 overexpression in tumorigenesis. Also,

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[3] We have previously cloned Aik, which is referred to as AIK1 in the present study, and AIK1 cDNA was resequenced and found to be identical to BTAK/aurora2.

[4] The abbreviations used are: BTAK, breast tumor amplified kinase; PCNA, proliferating cell nuclear antigen; ABC, avidin-biotin complex.

cell proliferation activity was estimated by counting PCNA-positive cells in breast cancer tissues.

Materials and Methods

Patients and Samples. Thirty-three female Japanese patients were diagnosed with breast carcinoma by biopsy and/or ultrasonography. All patients underwent radical mastectomy and axillary lymphadenectomy. Archival tissue was obtained from radical mastectomy specimens. Histopathologically, these breast cancers were all invasive ductal carcinomas: (*a*) 15 papillary carcinomas; (*b*) 6 medullary carcinomas; (*c*) 9 scirrhous carcinomas; and (*d*) 3 mucinous carcinomas. The breast samples consisted of excisional biopsy specimens of tissues, including six specimens of fibrocystic disease (two specimens with adenosis and four specimens with sclerosing adenosis), three specimens of fibroadenoma, and three specimens of intraductal papilloma. The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Three serial sections from each case were cut at 3 μm; one section was stained with H&E for histological examination, and the others were used for immunohistochemical staining against AIK1 protein and PCNA.

Production of a Polyclonal Antibody against AIK1. The antibody against AIK1 was raised and affinity-purified as described previously (*14*).

Immunohistochemistry. The ABC method was used to determine the localization of AIK1. Paraffin-embedded sections were dewaxed in xylene and rehydrated in a graded series of ethanol. After blocking endogenous peroxidase and biotin, the sections were incubated overnight with the primary antibody at 4°C (the antibody was diluted 100-fold). Next, the sections were incubated with a 100-fold dilution of biotinylated rat anti-rabbit IgG (Vectastain ABC Kit; Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 min. After an additional 60-min incubation with the ABC, the sections were reacted with 0.005% H_2O_2-3,3′-diaminobenzidine at room temperature for 90 s. Each incubation was followed by three washes with PBS. After staining with hematoxylin, the sections were examined under a light microscope. For each case, two negative controls were performed on serial sections. On one control section, the primary antibody was replaced with nonimmune serum, and on the other control section, incubation with the primary antibody was omitted. A semiquantitative evaluation was performed by two independent observers (T. T. and K. M.) to determine the AIK1 expression in the specimens. The expression was scored as follows: ++, high expression was detectable within the lesions; +, medium to high expression was detectable within the lesions; ±, expression was weakly detectable in part of the lesions; and −, expression was not detectable within the lesions.

The proliferative activity of invasive ductal breast carcinoma was determined by measuring PCNA-labeled nuclei. To determine the number of PCNA-labeled nuclei, deparaffinized sections (3-μm thick) were immunostained with the anti-PCNA monoclonal antibody PC10 (DAKO A/S, Glostrup, Denmark) as a primary antibody using the ABC method. All densely immunoreacted nuclei with PCNA were regarded as PCNA positive. Color photographs (×200) were taken from histologically representative areas (three fields/tumor, depending on the cellularity) of each breast carcinoma (12 papillary carcinomas, 6 medullary carcinomas, 4 scirrhous carcinomas, and 3 mucinous carcinomas). A minimum of 100 carcinoma cells/specimen was counted on the photographs for calculation of the PCNA labeling index. These immunoreactivities of the cancer cells were evaluated by two pathologists (K. M. and T. T.), and the mean of each two counts was considered as the PCNA labeling index.

Results

AIK1 Immunohistochemistry. Using an affinity-purified polyclonal rabbit antiserum recognizing human AIK1 protein, a moderate and predominant cytoplasmic AIK1 expression was detected in 31 of 33 (94%) invasive ductal breast carcinomas (Fig. 1). There was no preferential staining among the four histopathological types, indicating that AIK1 overexpression was independent of type (Table 1). In fibrocystic disease, weak expression was present in a part of the adenosis and the sclerosing adenosis. Similarly, the expression of AIK1 protein in intraductal papilloma and in the epithelial components of fibroadenoma was very weak and was only seen in certain parts. AIK1 was not stained in normal breast tissues, and necrotic cancer cells did not express the AIK1 protein.

PCNA Immunohistochemistry. All of the examined cancer tissues demonstrated a definite, positive nuclear staining for PCNA. No positive reaction was observed in the cytoplasm of carcinoma cells or on the negative control slides. The PCNA labeling indices in papillary, medullary, scirrhous, and mucinous carcinomas were 42.9 ± 13.3% (n = 12), 42.8 ± 10.8% (n = 6), 38.6 ± 11.6% (n = 4), and 30.3 ± 14.7% (n = 3), respectively. There was no statistical difference in the PCNA labeling index among the histological types.

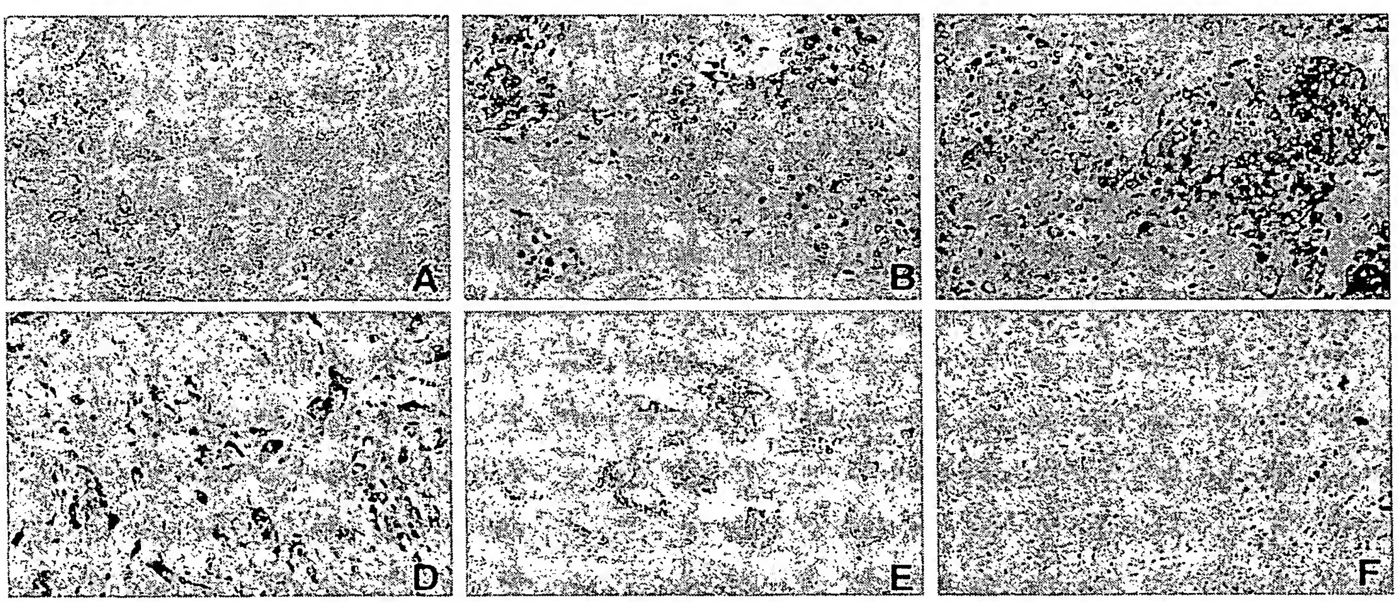

Fig. 1. Immunohistochemical staining for AIK1 in human breast tissue. *A–E*, tissues immunostained with AIK1 polyclonal antibody. *F*, control staining with nonimmune rabbit serum. No staining was achieved in nonneoplastic breast cancer tissue (fibrocystic disease; *A*). Strong AIK1 expression was found in invasive ductal breast carcinoma cells of papillary carcinoma (*B*), medullary carcinoma (*C*), scirrous carcinoma (*D*), and mucinous carcinoma (*E*). Control staining (*F*) was completely negative in all cases. Sections were counterstained with hematoxylin. Original magnification: *A* and *E*, ×16; *B–D* and *F*, ×40.

Table 1 *AIK1 expression in primary invasive breast carcinomas*

Pathological lesion	No. of cases	AIK1 expression			
		−	±	+	++
Normal tissue	6	6	0	0	0
Benign					
Adenosis	2	0	2	0	0
Sclerosing adenosis	4	0	4	0	0
Fibroadenoma	3	0	3	0	0
Intraductal papilloma	3	0	3	0	0
Carcinoma					
Papillary carcinoma	15	1	0	1	13
Medullary carcinoma	6	0	1	0	5
Scirrhous carcinoma	9	1	0	1	7
Mucinous carcinoma	3	0	0	0	3

Discussion

Altered expressions and/or mutations of cell cycle regulators result in the development of cancer (1, 34). Disruption of mitotic checkpoints can result in abnormal nuclei, missegregated chromosomes, and aneuploidy (35, 36). Among the most notable abnormalities commonly found in tumor cells are chromosomal rearrangements and changes in chromosome number (4–6). This property of cancer cells is important, especially for our understanding of the regulatory mechanisms that control the progression of malignancy. In colorectal tumors without microsatellite instability, for example, a defect in chromosome segregation results in gains or losses in excess of 10^{-2}/chromosome/segregation (6). Although the precise mechanisms by which duplicated chromosomes are equally segregated during mitosis are largely unknown, the centrosome is believed to play an important role(s) in the formation of bipolar spindles. Mutations in fly *aurora* and yeast *IPL1* are responsible for a chromosomal segregation defect, and the gene products encode putative serine/threonine kinases. AIK1 in human cells was also suggested to have a role in chromosome segregation and tumorigenesis (14, 16, 23).

In the present study, the majority (94%) of breast carcinomas with different histological types were found to overexpress AIK1 protein. To examine how AIK1 staining was related to cell proliferation, we stained these samples with another marker, PCNA. The mean PCNA labeling index was highest in papillary carcinoma (42.9%), followed by medullary carcinoma (42.8%), scirrhous carcinoma (38.6%), and mucinous carcinoma (30.3%). Less than half of the cancer cells were PCNA positive, whereas nearly all of the cancer cells were AIK1 positive in over 90% of the cases examined (Fig. 1). Positive staining with AIK1 at a high percentage does not merely seem to be an indication of cancer cell proliferation. Previous immunofluorescence studies revealed centrosomal localization of AIK1 during mitosis, but diffuse cytoplasmic staining was observed in the present study. Thus, it is conceivable that AIK1 overexpression is indicative of the pathological states of cancer cells.

To the best of our knowledge, this is the first immunohistochemical report showing that primary epithelial malignant cells overexpress centrosomal kinase AIK1, which was stained diffusely in cytoplasm. Our data in human breast cancer tissues are in agreement with those of a recent report showing that BTAK is overexpressed in human breast cancer cell lines (15). Members of the Aurora/Ipl1-related kinase family have a high degree of amino acid identity in their kinase domains and are involved in the regulation of the chromosome segregation process. Mutations in *aurora* of *Drosophila* and yeast *Ipl1* cause chromosome segregation abnormalities to generate polyploid and/or aneuploid nuclei to mitotic arrest (12, 13). The *AIK1* gene was mapped to human chromosome 20q13.2–13.3 (15, 33), and 20q13 amplification is common to many human malignancies (27–31), including breast (29, 37) and colorectal (16) cancers. Tanner *et al.* (38) suggested that the 20q13 amplification may define a subset of aggressive breast cancer. Breast cancer patients with aneuploid DNA reportedly have a poor prognosis (39). Therefore, how the expression of AIK1 protein in cancer tissues is involved in tumorigenesis is an important factor. Deregulation of Aurora/Ip11 family kinases in vertebrates, such as human AIK1 (14, 15), mouse STK1 (18) and Ayk1/IAK1 (19, 20), rat AIM-1 (21), and *Xenopus* pEg2 (23), may also contribute to polyploidy and/or aneuploidy in cancer cells. Tatsuka *et al.* (40) recently reported that the exogenously induced overexpression of wild-type AIM-1 in human diploid fibroblasts caused multinuclearity and aneuploidy. In addition to the findings of overexpression of AIK1 in human breast cancer cell lines (15), Bischoff *et al.* (16) have reported that the *BTAK* gene mapped to chromosome 20q13 is amplified and its mRNA is overexpressed in more than 50% of primary colorectal cancers. Recent investigations also found that overexpression of BTAK/AIK1 could amplify the centrosomes and transform rodent fibroblasts (16, 26). The results of the current study and those of other studies suggest that *AIK1* might be a potential oncogene in breast cancer, colon cancer, and possibly other solid malignancies.

The molecular mechanisms by which AIK1 protein is overexpressed in cancer cells have not been identified. Gene amplification of 20q13 has been reported in various cancers. Our preliminary semi-quantitative PCR experiments using DNA templates extracted from paraffin-embedded samples showed discrete amplification in 3 of 12 cases (data not shown). Compared to the percentage of AIK1 protein overexpression, the proportion of the cases with gene amplification is very low. Similar results were obtained by Zhou *et al.* (26) showing that 12% of primary breast cancers exhibited amplification of 20q13. They also reported cases with *BTAK/AIK1* mRNA overexpression without gene amplification, suggesting a rapid transcription or delayed degradation of its mRNA. We have previously noted the rapid degradation of AIK1 after the mitotic phase and the presence of destruction box-like sequences in AIK1, suggesting the possible involvement of ubiquitin-proteasome system in its degradation (14). It is conceivable that the prevention of protein degradation could also contribute to the AIK1 accumulation. Thus, in addition to gene amplification, other mechanisms by which AIK1 is overexpressed need to be studied.

It is to be noted that normal tissue was not stained with anti-AIK1 antibody, whereas all of the benign tumors examined were weakly stained in part. If the benign tumors were on the route to malignancy, it would be conceivable that overexpression of AIK1 might be one of the initial events to occur in the early stages of tumorigenesis. Additional studies are necessary to clarify the precise molecular relationship between AIK1 expression and the tumorigenicity, but it is tempting to postulate that AIK1 overexpression may cause abnormal centrosome function, abnormal spindle formation, and chromosome segregation, resulting in the aneuploidy observed in cancers.

In summary, we demonstrated immunohistochemically that the AIK1 protein is highly expressed in invasive ductal carcinoma of the breast. Disruption of the protein forming a centrosome-associated kinase cascade may lead to genomic instability and the chromosome segregation defect. The findings also suggest that overexpression of the protein may be of pathogenic and/or prognostic importance in breast cancer. Investigations of AIK1 expression in invasive lobular carcinomas and *in situ* carcinoma of ductal and lobular origin are now being considered. Additional studies using the antibody may provide a possible therapeutic tool for the treatment of breast cancer. In any event, further research is clearly warranted to identify the physiological substrate for the overexpressed AIK1 kinase in breast cancer and other cancer cells.

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